

**MOLECULAR ANALYSIS OF THE *VIBRIO ALGINOLYTICUS*  
SUCROSE UTILIZATION SYSTEM CLONED INTO  
*ESCHERICHIA COLI***

by

**Gregory Lloyd Blatch**

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy in the Faculty of Science, University of Cape Town

September 1990

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## ACKNOWLEDGEMENTS

Thank-you Professor Dave Woods for being an excellent supervisor; your patient guidance and positive attitude were indispensable.

A special thanks go to Associate Professor Frank Robb who made many helpful contributions at the start of this study, and to Professor Doug Rawlings who has always been available to give advice. I am deeply indebted to Dr Heide Goodman and Dr Val Abratt for their proof-reading skills which refined the raw thesis.

Thanks go to my colleagues and friends for providing a stimulating yet relaxed research environment. Especially Dr Renate Scholle who always gave critical and constructive advice, and Dave and Eldie Berger for support, friendship and good times.

A warm thanks to my wife, Heather, who has been a constant source of encouragement and moral support during these final years of study. I cannot thank my parents enough for their consistent spiritual support and financial assistance throughout my university career.

I gratefully acknowledge the financial support of the Foundation for Research Development, Council for Scientific and Industrial Research.

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September, 1990

ABSTRACT

This dissertation represents a continuation of the research on the sucrose utilization system of the aerobic, collagenolytic, halotolerant, Gram-negative bacterium *Vibrio alginolyticus*. The *V. alginolyticus* sucrose utilization system originally cloned into *Escherichia coli* on plasmid pVS100 involves a sucrose enzyme (gene *scrB*), and a sucrose uptake system. Synthesis of the sucrose enzyme and sucrose uptake system in *V. alginolyticus* and *E. coli*(pVS100) is regulated. The nucleotide sequence and analysis of DNA regions encoding the sucrose uptake and regulatory functions are presented here. An investigation of the expression of the *V. alginolyticus* sucrose utilization system in *Bacillus subtilis* is also presented.

The nucleotide (nt) sequence of the DNA fragment upstream of the *scrB* gene containing the *V. alginolyticus* sucrose uptake-encoding region was determined. This region contains two genes, *scrA* and *scrK*. The *scrA* gene encodes a sucrose uptake protein or enzyme II<sup>Sucrose</sup> (EII<sup>Scr</sup>) protein of the phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS). The *V. alginolyticus* sucrose utilization system was not expressed in *ptsH* and *crr* strains of *E. coli* indicating that sucrose transport required a functional PTS and was enzyme III<sup>Glucose</sup> (EIII<sup>Glc</sup>)-dependent. The deduced amino acid (aa) sequence for the *V. alginolyticus* EII<sup>Scr</sup> protein was homologous with the EII<sup>Scr</sup> proteins from *Streptococcus mutans*, *Salmonella typhimurium* (pUR400 system) and *B. subtilis*. Transposon *phoA* mutagenesis experiments indicated that the EII<sup>Scr</sup> protein was a

membrane-bound protein with a region that extended into the periplasm. The *scrK* gene encoded a fructokinase and the deduced aa sequence was homologous with the *E. coli* enzymes, 6-phosphofructokinase (isoenzyme 2) and ribokinase.

Nucleotide sequencing of the region upstream of the *V. alginolyticus scrA* gene located an Scr repressor-encoding gene (*scrR*) and an unidentified open reading frame (ORF5). Both the *scrR* gene and ORF5 were in the opposite orientation with respect to the *scrA* gene. The deduced aa sequence of the Scr repressor was homologous to the Gal, Lac and Cyt repressors of *E. coli* and contained a helix-turn-helix DNA binding domain. The codon usage of the *scrR* gene and ORF5 did not reflect the bias observed for 15 other *V. alginolyticus* genes. The *scrR* gene was shown to produce a protein in a DNA-directed cell-free coupled transcription-translation system. The *scrR* gene was required *in cis* for regulation of the *V. alginolyticus* sucrose utilization system in *E. coli*, and a deletion of the *scrR* gene could not be complemented *in trans*.

The sucrose utilization system of *V. alginolyticus* (*scrRAKB*) was subcloned into the *B. subtilis* shuttle vector pHV33 to generate pVS203, and transformed into *B. subtilis* QB602 (a *sacA sacB* mutant). The *V. alginolyticus* sucrose utilization system was not maintained in *B. subtilis* QB602 as the *V. alginolyticus* DNA was spontaneously deleted in *B. subtilis* QB602. Transformation of *B. subtilis* QB602 with pHV33 containing the *V. alginolyticus scrB* and *scrK* genes (pVS210) yielded 10-20% stable transformants which produced high levels of sucrase activity. These stable transformants may be the result of a putative recombination event between the *scrB* gene and the chromosomal *sacA* gene. The remaining 80-90% of the transformants produced low levels of sucrase activity. The low levels of sucrase production represented the actual level of expression of the *V. alginolyticus scrB* gene in *B. subtilis* QB602(pVS210).

## ABBREVIATIONS

A	adenine
aa	amino acids
Ala	L-alanine (A)
$A_{600}$	absorbance at 600 nm
Ap	ampicillin
Arg	L-arginine (R)
Asn	L-asparagine (N)
Asp	L-aspartic acid (D)
ATP	adenosine 5'-triphosphate
Bgl	(superscript) $\beta$ -glucosides
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CAP	catabolite activator protein
C-	carboxy- (terminal)
cAMP	adenosine-3',5'-cyclic monophosphate
CCCP	carbonyl cyanide- <i>m</i> -chlorophenyl hydrazone
Ci	Curie
Cm	chloramphenicol
CMM	C mineral medium
CsCl	caesium chloride
Cys	L-cysteine (C)
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
EDTA	ethylenediaminetetra-acetic acid
EI	enzyme I of the PTS
EII	enzyme II of the PTS
EIII	enzyme III of the PTS
EtBr	ethidium bromide
Fru	(superscript) fructose
g	standard gravitational acceleration
G	guanine
Glc	(superscript) glucose
Gln	L-glutamine (Q)
Glu	L-glutamic acid (E)
Gly	L-glycine (G)
Gut	(superscript) glucitol

h	hour(s)
His	L-histidine (H)
HPr	heat-stable protein of the PTS
Ile	L-isoleucine (I)
kb	kilobase pair(s)
kDa	kilodalton(s)
Km	kanamycin
Lac	(superscript) lactose
LB	Luria-Bertani broth
Leu	L-leucine (L)
Lys	L-lysine (K)
Man	(superscript) mannose
Met	L-methionine (M)
min	minute(s)
$M_r$	relative molecular mass
MM	minimal medium
mRNA	messenger RNA
Mtl	(superscript) mannitol
N-	amino- (terminal)
Nag	(superscript) N-acetyl-glucosamine
nm	nanometers
nt	nucleotide(s)
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
Phe	L-phenylalanine (F)
Pho	alkaline phosphatase
<i>phoA</i>	gene encoding alkaline phosphatase
PNPP	<i>p</i> -nitrophenyl phosphate
Pro	L-proline (P)
$p_R$	rightward promoter ( $\lambda$ )
PTS	phosphotransferase system
R	(superscript) resistance
RNA	ribonucleic acid
RNAse	ribonuclease

S	(superscript) sensitivity
s	second(s)
Scr	(superscript) sucrose
SDS	sodium dodecyl sulfate
Ser	L-serine (S)
Sor	(superscript) sorbose
T	thymine
TBE	Tris-borate EDTA buffer
TAE	Tris-acetate EDTA buffer
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
Thr	L-threonine (T)
Tn	transposon
Tris	tris(hydroxymethyl)aminomethane
Trp	L-tryptophan (W)
Tyr	L-tyrosine (Y)
UV	ultraviolet (light)
Val	L-valine (V)
v/v	volume/volume
w/v	weight/volume
XP	5-bromo-4-chloro-3-indolyl phosphate
::	novel joint (fusion)
()	designates plasmid-carrier state
$\alpha$	alpha
$\beta$	beta
$\Delta$	delta
$\lambda$	lambda
$\mu$	micro

## CHAPTER ONE

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## CHAPTER ONE

### GENERAL INTRODUCTION

#### 1.1 GENERAL INTRODUCTION TO *VIBRIO ALGINOLYTICUS*

##### 1.1.1 General characteristics

*Vibrio alginolyticus* is a Gram-negative, rod shaped, aerobic, halophilic bacterium found predominantly in marine coastal waters (Larsen and Farid, 1980). *V. alginolyticus*, originally classified as a biotype 2 of *Vibrio parahaemolyticus*, was reclassified as a separate species because of several different characteristics (Sakazaki, 1968). The main differences described were acetoin production, sucrose fermentation, growth in 10% NaCl and swarming on agar plates containing 2 to 7% NaCl. *V. parahaemolyticus* is negative for these characteristics and *V. alginolyticus* is positive for these characteristics. Within the last 15 years *V. alginolyticus* has been recognized as a human pathogen and has been isolated from infections of the ear, eye, hand, leg, lung, blood and burns which, in most instances, had been exposed to sea water (Joseph *et al.*, 1982).

A collagenolytic *V. alginolyticus* strain originally isolated from hides and shown to be the cause of leather decay (Welton and Woods, 1973; 1975) has been the subject of extensive research over the last 20 years. This *V. alginolyticus* strain was the source of the genetic material used in this study and therefore warrants a special introduction.

##### 1.1.2 Studies on a collagenolytic *V. alginolyticus* strain

Welton and Woods (1973; 1975) described the isolation from hides of an aerobic, halotolerant, collagenolytic, Gram-negative bacterium which was originally classified as an *Achromobacter iophagus* strain. The identification was originally

confirmed by the National Collection of Industrial Bacteria (Aberdeen, Scotland) but has since been reinvestigated by M. Hendrie (National Collection of Industrial Bacteria) and reclassified as a *V. alginolyticus* strain (NCIB 11038). Recently, the 5S rRNA sequence of this collagenolytic *V. alginolyticus* strain was shown to be similar to that of other marine *V. alginolyticus* strains (Scholle *et al.*, 1989).

**COLLAGENASE:** *V. alginolyticus* displayed strong extracellular collagenase activity when propagated in the presence of 2.34% (w/v) NaCl under aerobic conditions (Welton and Woods, 1975). Collagenase production by *V. alginolyticus* was recognized as industrially lucrative and it was proposed that *V. alginolyticus* would be more suitable for the commercial production of collagenase than *Clostridium histolyticum*, at that time the major industrial source of this enzyme (Welton and Woods, 1975). The extracellular collagenase displayed a higher specific activity than the *C. histolyticum* enzyme, and it was not associated with a lethal clostridial toxin (Welton and Woods, 1975; Lecroisey *et al.*, 1975; Keil-Dlouha *et al.*, 1976). The collagenase produced by *V. alginolyticus* was inducible by either collagen or its high molecular weight fragments, and by certain fragments in peptone (Difco), and was synthesized as the culture entered the stationary phase (Keil-Dlouha *et al.*, 1976; Robbertse *et al.*, 1978; Reid *et al.*, 1978, 1980). Collagenase synthesis in *V. alginolyticus* was subject to end-product repression and was repressed by various amino acids (aa) and ammonium ions. Collagenase synthesis in *V. alginolyticus* was also sensitive to catabolite repression by glucose and a number of carbon sources. Catabolite repression could not be relieved by the addition of cyclic nucleotides indicating that collagenase synthesis was not regulated by classical catabolite repression (Reid *et al.*, 1978). Collagenase production in *V. alginolyticus* was found to be affected by inhibitors of translation (chloramphenicol) and transcription (rifampin) in a similar way to exoenzyme production in late logarithmic phase cultures of *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Pseudomonas lemoignei* (Reid *et al.*, 1980). Inhibition of translation by chloramphenicol in *V. alginolyticus* prevented collagenase production whereas the inhibition of transcription by rifampin did not have an immediate effect on collagenase production, indicating that the collagenase may be translated from a reservoir or pool of mRNA, in the absence of transcription.

**PROTEASES:** In addition to collagenase *V. alginolyticus* was found to secrete alkaline serine proteases (Long *et al.*, 1981). Like collagenase synthesis, alkaline protease synthesis in *V. alginolyticus* was subject to catabolite and end-product repression and was repressed by various carbon sources, aa and ammonium ions (Long *et al.*, 1981). However in contrast to collagenase synthesis, alkaline protease synthesis did not require a specific inducer and was produced in tryptone or minimal media during the late stationary growth phase (Long *et al.*, 1981). This, together with the discovery that alkaline serine protease synthesis, but not collagenase synthesis, was stimulated by histidine and urocanic acid, implied that the two enzyme activities were under independent control (Long *et al.*, 1981; Bowden *et al.*, 1982). The stimulation of alkaline protease production in *V. alginolyticus* by histidine and urocanic acid suggested that the histidine utilization system (Hut) may be involved in the regulation of alkaline protease synthesis. *V. alginolyticus* has been found to be similar to *B. subtilis* in that in both bacteria histidine was the inducer of the Hut enzymes (Chasin and Magasanik 1968; Bowden *et al.*, 1982). The production of both collagenase and alkaline proteases in *V. alginolyticus* was found to be regulated by temperature and oxygen (Hare *et al.*, 1981). Optimal yields of these enzymes were obtained at 30°C and their production was significantly reduced by a temperature shift to 37°C or by the lack of oxygen.

The extracellular protease activity in *V. alginolyticus* cultures was found to involve 6 distinct alkaline serine proteases (Hare *et al.*, 1983; Deane *et al.*, 1986). Responses to temperature shifts and to the addition of histidine were caused primarily by the regulation of one (protease 1) of the 6 different proteases. Growth of *V. alginolyticus* in a complex proteinaceous medium resulted in the identification of a seventh SDS-resistant, Ca<sup>2+</sup>-dependent alkaline serine exoprotease (protease A). Protease A synthesis was not regulated by temperature, but its production was reduced in the absence of aeration (Deane *et al.*, 1987b). The gene encoding the protease A from *V. alginolyticus* was cloned in *E. coli* and was expressed from its own promoter in *E. coli* (Deane *et al.*, 1987a). Although active exoprotease was produced by late stationary phase recombinant *E. coli* cultures after 15 h incubation in proteinaceous medium containing Ca<sup>2+</sup>, transcription and translation of the exoprotease occurred before 6 h, during exponential growth. The cloned *V. alginolyticus* exoprotease was

synthesized as a pool of inactive precursor molecules during exponential growth, and released as active exoprotease 8 h later by a process which did not require protein synthesis or involve cell lysis. The nucleotide (nt) sequence of the gene encoding the *V. alginolyticus* protease A (*proA*) was determined (Deane *et al.*, 1989). The predicted aa sequence of the protease A showed significant homology with other serine proteases of the subtilisin family. It was interesting that the protease A from the Gram-negative *V. alginolyticus* showed stronger homology with the Gram-positive *Bacillus licheniformis* protease than with the alkaline serine protease produced by a more closely related Gram-negative *Serratia marcescens* strain (Deane *et al.*, 1989).

**NITROGEN METABOLISM:** Nitrogen metabolism has been investigated in *V. alginolyticus*. It was found that the nitrogen catabolic enzymes of *V. alginolyticus* were not regulated by nitrogen catabolite repression (Bodasing *et al.*, 1983). In this respect *V. alginolyticus* resembled *Bacillus* strains rather than the more closely related Gram-negative enteric bacteria where nitrogen catabolite repression has been shown to operate (Bodasing *et al.*, 1983). This finding initiated a study of the *V. alginolyticus* glutamine synthetase, since this enzyme plays a central role in ammonia assimilation. In contrast to the nitrogen catabolic enzymes, the *V. alginolyticus* glutamine synthetase was regulated in a similar manner to other Gram-negative bacteria; it was regulated by adenylation and was not subject to feedback inhibition by glutamine (Bodasing *et al.*, 1985). Glutamine synthetase synthesis in *V. alginolyticus* was regulated by temperature, oxygen and nitrogen levels (Maharaj *et al.*, 1986). The structural gene for the glutamine synthetase (*glnA*) of *V. alginolyticus* was cloned in *E. coli*, where it was shown to be regulated by temperature, oxygen and nitrogen levels (Maharaj *et al.*, 1986). The nt sequence of the cloned fragment containing the *V. alginolyticus* *glnA* gene was determined (Maharaj *et al.*, 1989). The structure of the *V. alginolyticus* *glnA* region was similar to those of *E. coli* and other enterobacteria and consisted of *ntrB* and *ntrC* genes linked to *glnA*. The predicted aa sequence of the *V. alginolyticus* glutamine synthetase subunit was found to be strongly homologous to the *E. coli* glutamine synthetase subunit.

## 1.2 CARBOHYDRATE TRANSPORT SYSTEMS IN BACTERIA

Nutrient acquisition is an important function of all living organisms, and many cellular activities are directed toward this goal. An integral aspect of the acquisition process is the transport of nutrients from the external environment through the cell barrier into the cytoplasm, where metabolism occurs. In Gram-negative bacteria the cell surface consists of 3 layers: an outer membrane, the cell wall proper or peptidoglycan, and an inner, or cytoplasmic membrane (Ames, 1986). The space between the inner and outer membranes is often referred to as the periplasm. Gram-positive bacteria differ fundamentally from Gram-negative bacteria in that they do not contain an outer membrane. Limited permeability to small solutes is available through the outer membrane by way of proteinaceous channels, which may be substrate non-specific (porins) or substrate-specific. The cell wall proper is commonly regarded as an entirely permeable layer, conferring rigidity to the cell while forming a widely open network through which nutrient diffusion readily occurs. The cytoplasmic membrane, on the other hand, is impermeable to almost every solute unless a special transport system is provided. Bacteria have evolved a variety of mechanisms by which solutes are transported into and out of cells. Carrier-mediated transport systems can be categorized into 4 groups according to the source of energy coupled to the transport process.

### **1.2.1 Facilitated diffusion**

Facilitated diffusion is a process not coupled to metabolic energy and is therefore not capable of accumulating a substrate against a concentration gradient. However, facilitated diffusion does require a specific membrane protein. Glycerol is the only carbohydrate known to be transported by facilitated diffusion (Dills *et al.*, 1980). The following systems are "active" or "concentrative" transport systems since they are energy coupled and accumulate substrates against concentration gradients.

### 1.2.2 Osmotic shock-sensitive active transport

Shock-sensitive transport systems in Gram-negative bacteria are so named because uptake via these systems is virtually abolished by the cold osmotic shock procedure (Neu and Heppel, 1965). Shock-sensitive systems have a complex organization being generally composed of 4 proteins of which one, the substrate receptor, is located in the periplasm and is thus released by an osmotic shock (Ames, 1986). The other three proteins are membrane-bound; two are very hydrophobic and embedded in the cytoplasmic membrane, whereas the third one has a hydrophilic sequence and is thought to be bound to the inner surface of the cytoplasmic membrane. Another distinguishing characteristic of shock-sensitive systems is the mechanism by which cellular energy is coupled to their concentrative power. It has been proposed that they utilize a chemical source of metabolic energy derived from substrate level phosphorylation (Dills *et al.*, 1980) as opposed to being driven by an electrochemical potential (used by transport systems in the next category). Recent work on the histidine and maltose transport systems of *E. coli* has suggested that these shock-sensitive systems have an obligatory requirement for ATP (or a closely related molecule) (Joshi *et al.*, 1989). Many shock-sensitive systems for carbohydrates (maltose, arabinose, ribose, and xylose) and aa (histidine, glutamine and leucine-isoleucine-valine) have been discovered in *E. coli* and *S. typhimurium* (Dills *et al.*, 1980; Ames, 1986).

### 1.2.3 Electrochemical potential-dependent active transport

The chemiosmotic theory formulated by Mitchell (1961) is now widely accepted to explain the principle mode of energy coupling in membranes. According to the theory, the respiratory electron transport chain is oriented across the membrane so that the oxidation of the electron carriers is accompanied by the translocation of protons in an outward direction. It further states that membranes are impermeable to protons. Hence, as a consequence of electron transport, a proton gradient or electrochemical potential difference of  $H^+$  (proton motive force) is created across the membrane. Thus the accumulation of a carbohydrate in the cell can be coupled to the movement of protons down a concentration gradient. Three terms have been

proposed to define the mechanisms by which metabolites are coupled to ion transport: symport, antiport and uniport. The term symport describes a system in which two substrates are transported in the same direction as the carrier. Antiport systems are those in which two substrates are translocated in opposite directions. Uniport is defined as a process in which a solute moves across a membrane but is not tightly coupled to the movement of another species. It is now firmly established that the proton motive force can drive energy-dependent reactions such as active transport of solutes, ATP synthesis, or flagella motility in bacteria (Tokuda, 1989). These reactions are coupled to the back flow of  $H^+$  through the respective machinery. The transport of lactose by *E. coli* has been extensively studied and shown to be driven by the proton motive force (West, 1970; Wright *et al.*, 1986; Kaback *et al.*, 1990). Lactose uptake in *E. coli* is catalyzed by an  $H^+$ : $\beta$ -galactoside symport located in the cytoplasmic membrane. The protein, also called the lactose permease, is the product of the *lacY* gene of the *lac* operon. In *E. coli* proton-coupled transporters have also been found for galactose (*galP* gene product), arabinose (*araE* gene product) and xylose (*xylE* gene product) (Wright *et al.*, 1986; Maiden *et al.*, 1987).

It is now well established that  $Na^+$ , instead of  $H^+$ , can be used as the energy-coupling ion for a wide variety of secondary carbohydrate and aa transport systems. At least one, the melibiose permease (*melB* gene product), appears to be an intermediate between  $H^+$ - and  $Na^+$ -coupled systems (Tsuchiya *et al.*, 1982). The melibiose permease accepts  $Na^+$  and  $Li^+$  in addition to  $H^+$  for the transport of some galactosides. Various processes have been shown to establish an electrochemical potential difference of  $Na^+$  (sodium motive force) across the membrane.

**$Na^+/H^+$  ANTIPORTERS:** An  $Na^+/H^+$  antiporter is a carrier that catalyzes the exchange of  $Na^+$  and  $H^+$ , thereby translocating  $Na^+$  and  $H^+$  in opposite directions across a membrane (Krulwich, 1983).  $Na^+$  antiporters are envisaged as secondary active transport systems rather than primary pumps. This system has been shown to operate in the halophilic bacteria *Halobacterium halobium* and *Paracoccus halodenitrificans* and the marine bacterium *Alteromonas haloplanktis* (Krulwich, 1983; Dibrov *et al.*, 1986; Unemoto and Hayashi, 1989)

**Na<sup>+</sup>-ATPases:** Na<sup>+</sup>-ATPases extrude Na<sup>+</sup> at the expense of ATP and are primary pumps. Na<sup>+</sup>-ATPases have been detected in *Propionigenium modestum*, *Mycoplasma mycoides* and *Streptococcus faecalis* (Dibrov *et al.*, 1986; Rosen, 1986).

**Na-TRANSLOCATING DECARBOXYLASES:** Biotin-dependent decarboxylases are members of a class of enzymes involved in fermentation of organic acids. Several organic acid decarboxylases have recently been shown to be primary sodium pumps. Growth of *Klebsiella aerogenes* on citrate induces oxaloacetate decarboxylase which is membrane-bound and requires both sodium and biotin (Dimroth, 1982). The oxaloacetate decarboxylase creates a Na<sup>+</sup> electrochemical gradient with concomitant decarboxylation of oxaloacetate to pyruvate. Similar systems using glutaconyl CoA decarboxylases have been reported for *Acidaminococcus fermentans*, *Peptococcus aerogenes*, and *Clostridium symbiosum* (Rosen, 1986).

**A RESPIRATORY-DRIVEN Na<sup>+</sup> PUMP:** It was found that a marine *V. alginolyticus* strain contained an uncoupler-resistant respiratory-driven Na<sup>+</sup> pump (Tokuda and Unemoto, 1982; Tokuda and Unemoto, 1983; Tokuda, 1989; Unemoto and Hayashi, 1989). Two different types of pH dependent energetics were shown to be responsible for the generation of a Na<sup>+</sup> electrochemical potential in this marine *V. alginolyticus* strain. At acidic external pH the Na<sup>+</sup> electrochemical potential was established by a Na<sup>+</sup>/H<sup>+</sup> antiport system driven by the proton motive force generated as the immediate result of respiration. The generation of the Na<sup>+</sup> electrochemical potential, under these conditions was secondary to the generation of the proton motive force. At alkaline pH, however, the generation of the Na<sup>+</sup> electrochemical potential was a primary process, performed by a respiratory-driven Na<sup>+</sup> pump. Therefore, at acidic and alkaline pH's, significant differences were observed in the sensitivity of certain energy-linked processes to the proton conductor carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) which causes the collapse of a proton motive force. The active transport of several solutes, driven by the electrochemical potential of Na<sup>+</sup>, was resistant to CCCP at alkaline pH, but not at acidic pH. It was proposed that H<sup>+</sup> circulation might not be required for the growth of *V. alginolyticus* at alkaline pH, since the sodium motive force took

its place for membrane-linked energy requirements. This primary respiratory-driven  $\text{Na}^+$  pump has been shown to be catalyzed by a  $\text{Na}^+$ -dependent NADH:quinone oxidoreductase (Unemoto and Hayashi, 1989). It was found for this marine *V. alginolyticus* strain that the accumulation of 19 aa and sucrose was driven by the  $\text{Na}^+$  electrochemical potential established by this primary respiratory-driven  $\text{Na}^+$  pump (Tokuda and Unemoto, 1982; Kakinuma and Unemoto, 1985).

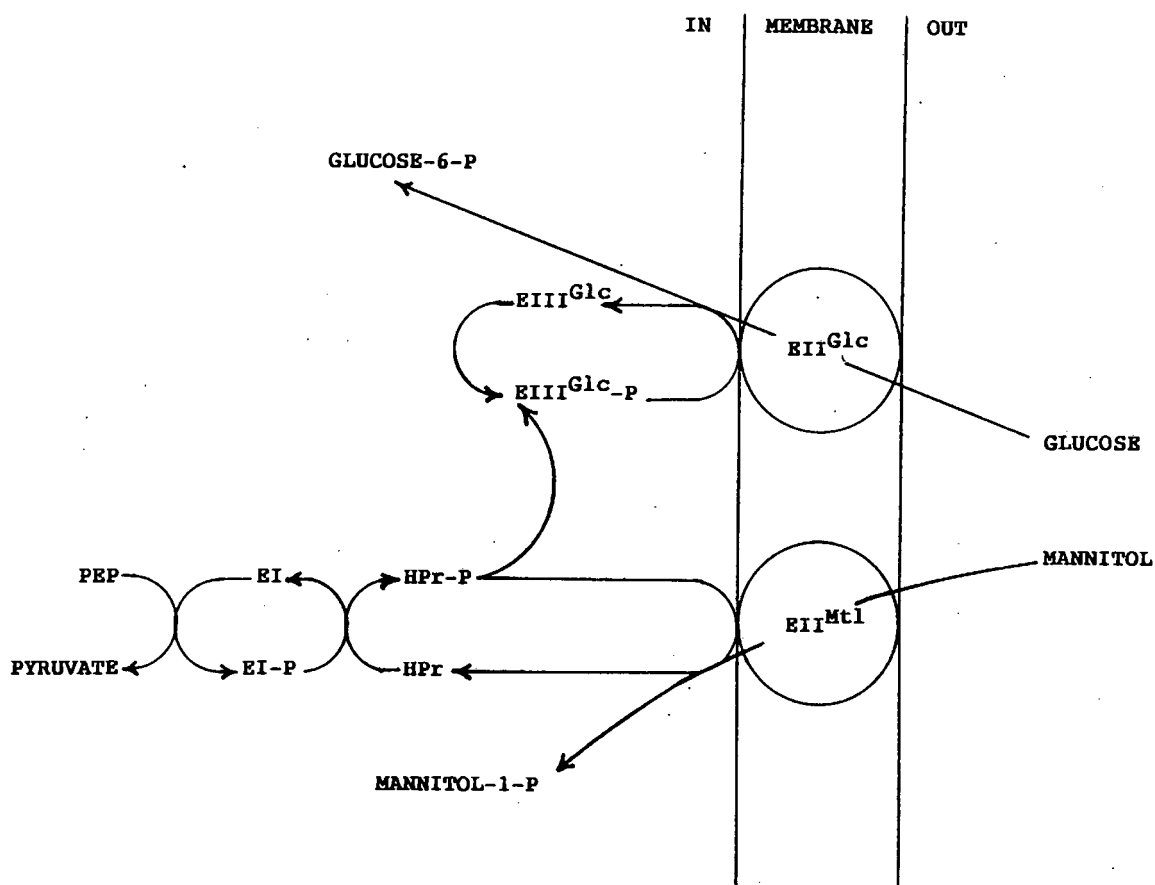
#### 1.2.4 Group translocation

Group translocation requires energy but is not strictly an active transport process because the transported compound is not concentrated unchanged (Lin *et al.*, 1984). Group translocation requires a specific carrier which modifies the substrate chemically during translocation and then releases the product into the cytoplasm. Once the substrate is modified the product can no longer pass the membrane barrier and is thus trapped inside the cell. In this process the metabolic energy is expended at the modification step. Anaerobic and facultatively anaerobic bacteria often transport carbohydrates by a process that delivers carbohydrate phosphates into the cytoplasm. The carbohydrate phosphate is the first intermediate in subsequent catabolism, thus providing a tight linkage between uptake and metabolism. This system, referred to as the phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS), has been extensively investigated and will be discussed in depth.

### 1.3 THE PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM

The PTS was discovered in cell extracts of *E. coli* as a system which catalyzed the phosphorylation of a number of carbohydrates with PEP as the phosphoryl donor (Kundig *et al.*, 1964). It soon became evident from genetic, transport and growth studies that this enzymatic activity represented part of a bacterial carbohydrate transport system (Postma and Roseman, 1976). Several protein fractions, both

soluble and membrane-bound, are now known to be involved in the transport and concomitant phosphorylation of carbohydrates by the PTS (Postma and Lengeler, 1985; Postma, 1987) (Fig. 1.1).



**FIG. 1.1** The phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS). Enzyme I (EI) and heat-stable protein (HPr) are the general PTS proteins. Of the many different enzyme II proteins, only two are shown. Enzyme II<sup>Mannitol</sup> ( $EII^{Mtl}$ ) is specific for mannitol, and enzyme II<sup>Glucose</sup> ( $EII^{Glc}$ ) together with enzyme III<sup>Glucose</sup> ( $EIII^{Glc}$ ) is specific for glucose. EI-P, HPr-P and  $EIII^{Glc-P}$  are the phosphorylated forms of EI, HPr and  $EIII^{Glc}$ , respectively (after Postma, 1987).

The two soluble proteins, enzyme I (EI) and heat-stable protein (HPr), are the general components of the PTS and are not substrate specific (Fig. 1.1). Substrate specificity of the PTS resides in the membrane-bound enzyme II (EII) proteins, each of which can recognize a series of structurally related carbohydrates (Fig. 1.1). During translocation of a substrate through the membrane a phosphoryl group is

initially transferred from PEP through the intermediate of phospho-EI to HPr, resulting in the production of phospho-HPr (Fig. 1.1). The phosphoryl group is next transferred from phospho-HPr to the substrate, by means of a phospho-EII intermediate (as for EII<sup>Mtl</sup> in Fig. 1.1). In a number of cases another soluble protein, enzyme III (EIII), is positioned between phospho-HPr and a particular EII (as for EII<sup>Glc</sup> and EIII<sup>Glc</sup> in Fig. 1.1). Therefore EII proteins can be categorized as either EIII-independent or EIII-dependent (forming an EII/EIII pair).

The genes encoding the various PTS components have been investigated in depth for *E. coli* and *S. typhimurium* (Postma and Lengeler, 1985; Postma, 1987; de Reuse *et al.*, 1989). The genes coding for the EII/EIII pairs are often linked. These genes and the genes encoding the EIII-independent EII proteins are scattered throughout the chromosome. They generally constitute operons comprising genes encoding enzymes involved in the first steps of the catabolism of the corresponding carbohydrate. The genes encoding the common proteins HPr and EI, *ptsH* and *ptsI* respectively, are clustered in an operon together with the *crr* gene encoding EIII<sup>Glc</sup>. The contiguity of the *crr* gene and the *ptsHI* genes appears to be related to the important regulatory functions of EIII<sup>Glc</sup> (discussed in section 1.3.2).

### 1.3.1 Components of the PTS

**SOLUBLE COMPONENTS ENZYME I AND HPr:** EI and HPr are both phosphorylated during carbohydrate transport. The phosphoryl group is bound to a histidine residue in both cases, *via* the N-3 position of the imidazole ring in EI and *via* the N-1 position in HPr (Postma, 1987; Bramley and Kornberg, 1987). EI has been purified from *E. coli* (Robillard *et al.*, 1979) and *S. typhimurium* (Weigel *et al.*, 1982) and the active enzyme found to consist of two identical polypeptides, the molecular weights of which have been estimated to be between 58 000 and 70 000. The *ptsI* gene from *E. coli* has been cloned and the nt and deduced aa sequences for EI determined (Saffen *et al.*, 1987). EI has been purified to homogeneity from *Staphylococcus aureus*, *S. faecalis* and *Streptococcus lactis* (Reizer *et al.*, 1988). The active site peptides from *S. lactis* and *S. faecalis* have been sequenced and comparison with

the primary structure of EI of *E. coli* showed homology around histidine 189 (Hengstenberg *et al.*, 1989).

The aa sequence of the *S. typhimurium* HPr was determined using the purified enzyme (Powers and Roseman, 1984). The *ptsH* gene of *E. coli* has been sequenced and the deduced aa sequence for the HPr determined (de Reuse *et al.*, 1985). Comparison of the aa sequences for the HPr's from *E. coli* and *S. typhimurium* revealed that they were identical. Both HPr's are small proteins consisting of 85 aa and with a relative molecular mass ( $M_r$ ) of approximately 9 000.

The *ptsH* gene from *B. subtilis* has been sequenced and the deduced aa sequence for the HPr determined (Gonzy-Treboul *et al.*, 1989). The aa sequences for the purified HPr's from *S. aureus*, *Staphylococcus carnosus* and *S. faecalis* have been determined and showed significant homology to one another and to the deduced aa sequence for the *B. subtilis* HPr (Hengstenberg *et al.*, 1989). The HPr's from these Gram-positive bacteria were compared to the HPr from Gram-negative *E. coli* (Reizer *et al.*, 1988; Deutscher *et al.*, 1989; Hengstenberg *et al.*, 1989; Reizer, 1989). All HPr's showed significant homology around a conserved histidine residue (His15), which was conserved in all the proteins and has been shown to be the PEP-dependent phosphorylation site. The Gram-positive HPr's also showed significant homology around a conserved serine residue (Ser46) which has been shown to be the site of ATP-dependent phosphorylation by a HPr kinase. This serine residue was also present in the Gram-negative HPr but homology around Ser46 was limited, and could explain why the HPr of *E. coli* has been shown to not be a substrate for the Gram-positive HPr-kinase.

**ENZYME II AND ENZYME II/ENZYME III PAIRS:** Sequence data is now available for a number of different EIII-independent EII proteins or EII/EIII pairs (Table 1.1). Each of these appears to be phosphorylated twice during transport and concomitant phosphorylation of their specific carbohydrate; firstly by means of HPr, at a histidine residue located in either the EIII protein or in the C-terminal domain of the EIII-independent EII protein, and secondly on a residue located in the membrane associated domain of either the EIII-dependent EII protein or the EIII-independent

EII protein (Saier *et al.*, 1988; Lengeler *et al.*, 1990). A number of the large EIII-independent EII proteins consist of a single polypeptide of 625 to 675 aa residues, almost exactly the sum of the smaller EIII-dependent EII proteins plus their corresponding EIII proteins (EII<sup>Nag</sup> and EII<sup>Glc</sup>/EIII<sup>Glc</sup> and EII<sup>Bgl</sup> and EII<sup>Scr</sup>/EIII<sup>Glc</sup>; Table 1.1) (Saier *et al.*, 1988). Note that for *K. pneumoniae* and *S. typhimurium* (pUR400 system) the EII<sup>Scr</sup> proteins have been found to interact with EIII<sup>Glc</sup> (EII<sup>Scr</sup>/EIII<sup>Glc</sup> pair) (Lengeler *et al.*, 1982; Lengeler *et al.*, 1990) while the EII<sup>Scr</sup> protein of *S. mutans* appears to be an EIII-independent EII<sup>Scr</sup> (Sato *et al.*, 1989) (see section 1.4).

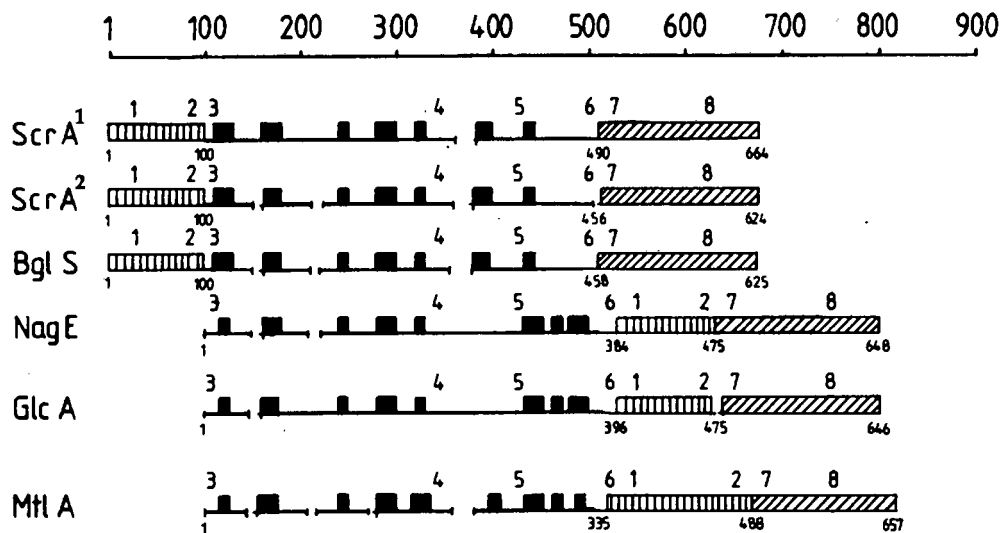
TABLE 1.1 Size comparisons of the different EII proteins and the EII/EIII pairs (after Lengeler *et al.*, 1990).

PTS <sup>a</sup>	Origin	Number of Subunits	Number of aa Residues		
			EII	EIII	T <sup>b</sup>
Scr	pUR400	2	456	169	625
Scr	<i>K. pneumoniae</i>	2	456	169	625
Sac	<i>B. subtilis</i>	2	460	?	?
Scr	<i>S. mutans</i>	1	664	-	664
Bgl	<i>E. coli</i>	1	625	-	625
Glc	<i>E. coli</i>	2	477	169	646
Nag	<i>E. coli</i>	1	648	-	648
Nag	<i>K. pneumoniae</i>	1	651	-	651
Mtl	<i>E. coli</i>	1	637	-	637
Mtl	<i>S. carnosus</i>	?	?	140	?
Gut	<i>E. coli</i>	2	506	123	629
Lac	<i>S. aureus</i>	2	572	103	675
Lac	<i>L. casei</i>	?	?	103	?
Fru	<i>E. coli</i>	2	563	376	939
Man	<i>E. coli</i>	3	266,283	323	1421
Sor	<i>K. pneumoniae</i>	?	?	137+182	?

<sup>a</sup> Each PTS is categorized according to the specific carbohydrate transported: Scr/Sac=sucrose, Bgl= $\beta$ -glucosides, Glc=D-glucose, Nag=N-acetyl-glucosamine, Mtl=D-mannitol, Gut=D-glucitol, Lac=lactose, Fru=D-fructose, Man=D-mannose, Sor=L-sorbose.

<sup>b</sup> T=sum of the aa residues for the EII and EIII proteins.

Analysis of the primary structure of the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup>/EIII<sup>Glc</sup> pair, the *S. mutans* EII<sup>Scr</sup> protein, the *B. subtilis* EII<sup>Scr</sup> protein and the *E. coli* EII<sup>Bgl</sup>, EII<sup>Nag</sup>, EII<sup>Mtl</sup> proteins and EII<sup>Glc</sup>/EIII<sup>Glc</sup> pair has revealed three large domains (Bramley and Kornberg, 1987; Ebner and Lengeler, 1988; Robillard and Lolkema, 1988; Saier *et al.*, 1988; Lengeler *et al.*, 1990). Lengeler *et al.* (1990) has categorized these domains into a hydrophobic domain, a hydrophilic EIII-like domain and a hydrophilic EIII-binding domain (Fig. 1.2).



**FIG. 1.2** Structural domains common to several EII proteins: *S. mutans* EII<sup>Scr</sup> protein (ScrA<sup>1</sup>); *S. typhimurium* EII<sup>Scr</sup>/EIII<sup>Glc</sup> pair (ScrA<sup>2</sup>); *E. coli* EII<sup>Bgl</sup> protein (BglS), EII<sup>Nag</sup> protein (NagE), EII<sup>Glc</sup>/EIII<sup>Glc</sup> pair (GlcA) and EII<sup>Mtl</sup> protein (MtlA). The hydrophobic domain with strongly or less strongly membrane seeking structures (wider or narrower solid blocks), EIII-like domain (diagonally hatched blocks) and EIII-binding domain (vertical hatched blocks) are indicated. The scale above the sequences indicates from 1 to 900 aa residues length, given also for each sequence (numbers immediately below a sequence). Other numbers indicate highly conserved structures common to all EII proteins listed: 1, phosphoryl-cysteine, acceptor from 8 and probable donator to the substrate; 2, 6 and 7, sequences enriched for charged aa; 3, amphipathic helix; 4, conserved histidine residue; 5, GITE motif; 8, phosphoryl-histidine acceptor from HPr and donator to 1 (after Lengeler *et al.*, 1990).

The hydrophobic domains of the EII proteins analyzed were found to be about 350 to 380 aa in length. These regions were apparently the membrane-bound parts of the proteins and did not show a high degree of homology between the various EII proteins. However a few characteristic structures were found (structures 3-6 in Fig. 1.2) (Lengeler *et al.*, 1990). Each hydrophobic domain started with an

amphipathic helix (structure 3 in Fig. 1.2) and contained a highly conserved histidine residue (structure 4 in Fig. 1.2) and GITE motif (structure 5 in Fig. 1.2). The hydrophobic domain, whether remaining free or fused to another domain, invariably ended with a series of charged aa (structure 6 in Fig. 1.2).

The EIII-like domains of the EII proteins analyzed were found to be hydrophilic domains of about 170 aa in length. This domain was proposed to be either the free EIII protein encoded by a distinct gene (*crr* for EIII<sup>Glc</sup> which interacted with EII<sup>Glc</sup> and EII<sup>Scr</sup>), or an EIII-like domain covalently bound to the EII protein (EII<sup>Nag</sup> and EII<sup>Bgl</sup>). The histidine residue phosphorylated by phospho-HPr in EIII<sup>Glc</sup> of *E. coli* has been located (His91; structure 8 in Fig. 1.2) (Dorschug *et al.*, 1984). It has been shown that the EIII-like domains of the EII<sup>Nag</sup> and EII<sup>Bgl</sup> proteins, which at the aa level showed good homologies with EIII<sup>Glc</sup>, were the functional equivalent of this protein. It was found that the EIII-like domain of EII<sup>Nag</sup> and EII<sup>Bgl</sup> could replace EIII<sup>Glc</sup> in EII<sup>Glc</sup>-dependent and EII<sup>Scr</sup>-dependent glucose and sucrose transport and phosphorylation, respectively (Vogler *et al.*, 1988; Lengeler *et al.*, 1990). It was proposed that, as has been shown for EIII<sup>Glc</sup>, the phosphoryl group from phospho-HPr would be transferred to a histidine residue in the EIII-like domains of EII<sup>Nag</sup> (His569) and EII<sup>Bgl</sup> (His574), the structural equivalent of the phosphorylated His91 of EIII<sup>Glc</sup> (Lengeler *et al.*, 1990). This phosphoryl group would then be transferred either in an intermolecular reaction (EII<sup>Glc</sup>/EIII<sup>Glc</sup> and EII<sup>Scr</sup>/EIII<sup>Glc</sup>) or an intramolecular reaction (EII<sup>Nag</sup> and EII<sup>Bgl</sup>) to a second site on the respective EII proteins. This second phosphorylation site was initially proposed to be the highly conserved histidine residue located in the hydrophobic domain of all EII proteins (structure 4 in Fig. 1.2) (Saier *et al.*, 1988). However, it has recently been proposed that a conserved region in the EIII-binding domain (see below), containing a highly conserved cysteine residue, may contain the second phosphorylation site (Lengeler *et al.*, 1990).

The EIII-binding domains of the EII proteins analyzed were found to be hydrophilic domains of about 80 to 100 aa in length. This domain was shown to contain a highly conserved sequence and was located either at the N-terminus (EII<sup>Scr</sup> and EII<sup>Bgl</sup>) or at the C-terminus (EII<sup>Glc</sup> and EII<sup>Nag</sup>). Alignment of the aa sequences of this domain

for 8 EII proteins, revealed a clear consensus sequence centered around a highly conserved cysteine residue (Lengeler *et al.*, 1990) (structure 1 in Fig. 1.2). In addition, this domain appeared to have a characteristic accumulation of charged residues at its C-terminus (structure 2 in Fig. 1.2). It has been proposed that since this region was so highly conserved in the different EII proteins, it was the region which interacted with EIII<sup>Glc</sup> or EIII-like domains, receiving a phosphoryl group (probably *via* the highly conserved cysteine) and donating it to the incoming substrate (Lengeler *et al.*, 1990). In addition, a number of experimental findings appeared to support this proposal. For EII<sup>Glc</sup> this highly conserved cysteine residue (Cys421) has been shown to be the only catalytically essential cysteine residue (Meins *et al.*, 1988; Nuoffer *et al.*, 1988). Elegant experiments analyzing deletion mutations in the cloned *mtlA* gene of *E. coli* (Grisafi *et al.*, 1989; Jacobson and Stephan, 1989) and investigating complementation of C-terminal EII<sup>Mtl</sup> deletion proteins (White and Jacobson, 1990) defined 2 functional subdomains of the C-terminal hydrophilic half of EII<sup>Mtl</sup> (as shown in Fig 1.2). The first region (including residues 377 to 519) was equivalent to, but not significantly homologous to, the cysteine-containing EIII-binding domain of the other EII proteins and also contained an equivalent cysteine residue (Cys384). This cysteine residue (Cys384) was shown to be the immediate phosphoryl donor to mannitol. The second region resided in the extreme C-terminus and contained the critical histidine residue (His554) proposed to be phosphorylated by phospho-HPr and then to donate its phosphoryl group to Cys384 (equivalent to the EIII-like domain discussed above).

The N-termini of a number of EII proteins have been analyzed for the presence of amphipathic sequences by Saier *et al.* (1989). It was found that the EII<sup>Bgl</sup> protein of *E. coli* and the EII<sup>Scr</sup> proteins of *S. typhimurium* (pUR400 system), *B. subtilis* and *S. mutans* contained N-terminal amphipathic sequences capable of forming helices of large hydrophobic moments. These proteins therefore contain amphipathic sequences at their N-termini (start of the EIII-binding domain) and at the start of their hydrophobic domains. It was proposed that these amphipathic sequences play a role in targeting EII proteins to the cytoplasmic membrane (Saier *et al.*, 1989).

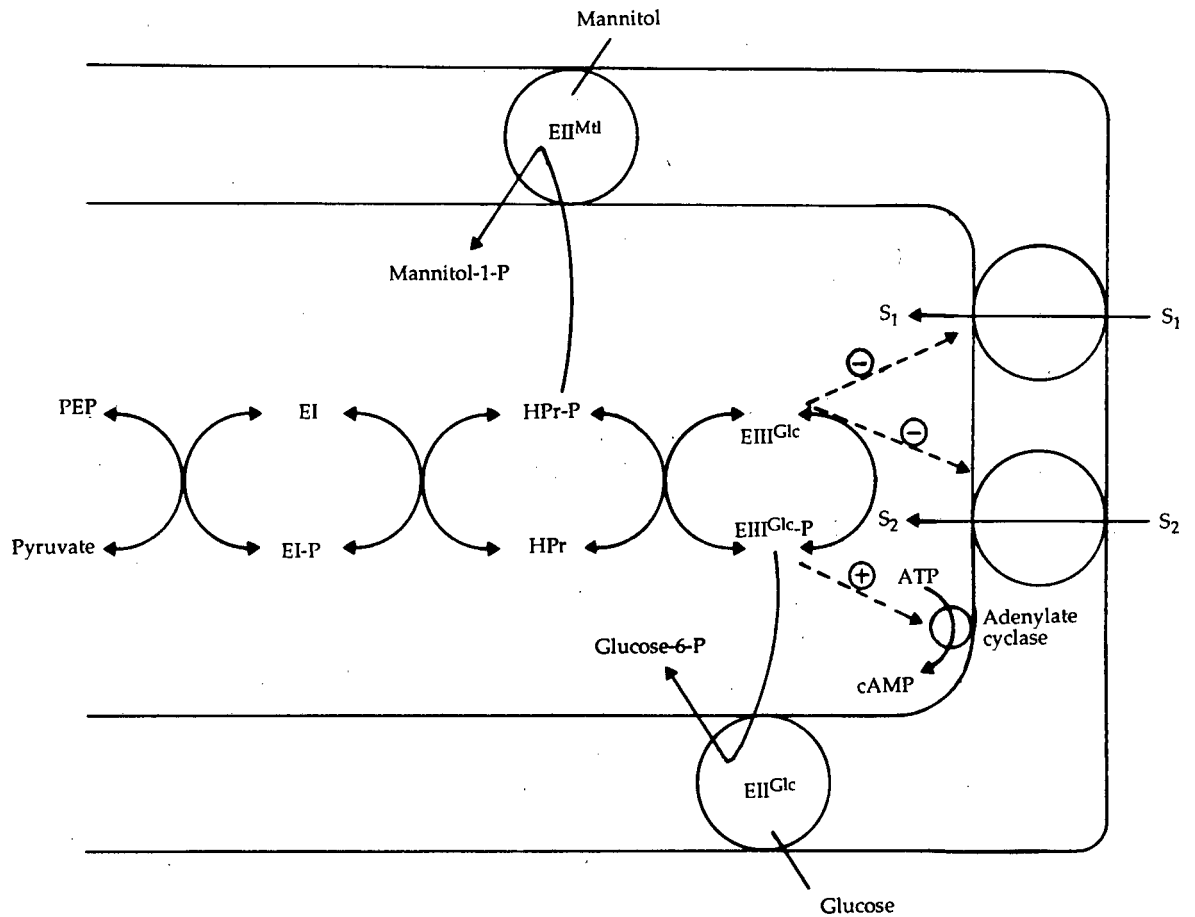
### 1.3.2 Regulation of carbohydrate metabolism and the PTS

**REGULATION OF THE PTS:** The PTS is regulated in a sophisticated way both at the level of enzyme synthesis and enzyme activity. In general for the enteric bacteria, EI and HPr are synthesized constitutively, whereas most EII and EIII proteins are inducible (Postma and Lengeler, 1985). Induction of the synthesis of several EII proteins is controlled in a negative way by highly specific repressors. The synthesis of EII proteins is not only controlled by induction but also by catabolite repression. Except for the *E. coli* EII proteins EII<sup>Glc</sup>, EII<sup>Man</sup> and EII<sup>Mtl</sup>, the synthesis of all other EII proteins in the enteric bacteria is sensitive to catabolite repression (Postma and Lengeler, 1985). Even the former EII proteins are not completely insensitive, since their synthesis is lowered in *cya* (gene product adenylate cyclase) and *crp* (gene product catabolite activator protein [CAP]) mutants.

Regulation at the level of enzyme activity is mediated effectively through various regulatory mechanisms which affect EII activity (Dills *et al.*, 1980; Postma and Lengeler, 1985; Postma, 1987). These include: (i) inhibition due to competition between two structurally related sugars for the substrate recognition site of an EII protein, (ii) inhibition of carbohydrate uptake by intracellular sugar phosphates, (iii) chemiosmotic control of transport activity due to responsiveness of the EII protein to the membrane potential and/or the intracellular pH, and (iv) inhibition of the uptake of one sugar by another sugar due to competition of the corresponding EII proteins for the common phospho-protein, phospho-HPr.

While the PTS is itself regulated in a sophisticated manner, it is also of great importance in the regulation of the peripheral catabolic pathways involving non-PTS carbohydrates. Central to this regulation in Gram-negative bacteria is the phosphorylation state of EIII<sup>Glc</sup>. EIII<sup>Glc</sup>-mediated regulation has not been demonstrated in Gram-positive bacteria. In Gram-positive bacteria another mode of regulation has been discovered in which the phosphorylation state of HPr plays a central role.

**EIII<sup>Glc</sup>-MEDIATED REGULATION:** When Gram-negative bacteria are exposed to two different carbon sources, one is frequently used preferentially and growth occurs in two phases (diauxie). In general a PTS sugar such as glucose is preferred by cells that exhibit diauxie, and when glucose is present in the medium, the PTS represses induction of many non-PTS operons and/or prevents the uptake of inducing molecules by decreasing the activity of the corresponding transport systems or metabolic enzymes (inducer exclusion) (Saier, 1977). An understanding of this complicated mode of regulation was gained through studies on mutants defective in components of the PTS. *E. coli* and *S. typhimurium* mutants lacking EI (*ptsI*) and HPr (*ptsH*) cannot grow on PTS carbohydrates as expected. But in addition, these mutants fail to grow on a number of non-PTS carbon sources (Saier *et al.*, 1976). For the enteric bacteria, non-PTS carbon sources that do not support the growth of *ptsH* and *ptsI* mutants have been divided into 2 classes (Postma, 1982). Class I includes well known non-PTS carbohydrates such as lactose, maltose, melibiose and glycerol. Class II consists of Krebs cycle intermediates (succinate, malate, and citrate), xylose, rhamnose and galactose. A number of critical observations led to a better understanding of how the PTS was involved in controlling the catabolism of non-PTS carbohydrates: (i) it was found that *ptsH* and *ptsI* mutants had similar phenotypes to *cya* and *crp* mutants, (ii) the phenotype of *ptsH* and *ptsI* mutants with respect to growth on non-PTS carbohydrates (class I and class II) could be reversed by the addition of exogenous adenosine-3',5'-cyclic monophosphate (cAMP) and (iii) suppressor mutations that restored growth of *ptsH* and *ptsI* mutants on class I non-PTS carbohydrates were found to be mutations that mapped in the *crr* gene thereby eliminating EIII<sup>Glc</sup> (Postma and Lengeler, 1985). Together these results suggested that the PTS was involved in the regulation of cAMP metabolism and that EIII<sup>Glc</sup> had an important role in this regulation. On the basis of these findings and other results, a model (Fig. 1.3) has been formulated in which the phosphorylation state of EIII<sup>Glc</sup> plays a central regulatory role (Postma and Lengeler, 1985; Postma, 1987; Postma *et al.*, 1989; Saier, 1989; Roseman and Meadow, 1990).



**FIG. 1.3** Model for the regulation by the PTS. In addition to the general proteins of the PTS, two EII proteins are shown, specific for mannitol (EII<sup>Mtl</sup>) and glucose (EII<sup>Glc</sup>). Activation (+) of adenylate cyclase by phospho-EIII<sup>Glc</sup> (EIII<sup>Glc</sup>-P) and inhibition (-) of two different non-PTS uptake systems by EIII<sup>Glc</sup> are indicated. S<sub>1</sub> and S<sub>2</sub> represent lactose, melibiose, maltose and glycerol (after Postma, 1987).

Two processes are postulated to be instrumental in regulation by the PTS. In one process non-phosphorylated EIII<sup>Glc</sup> forms a complex with the transport systems for class I carbohydrates (referred to as S<sub>1</sub> and S<sub>2</sub> in Fig. 1.3), thereby inhibiting their activity and preventing entry of class I carbohydrates. This proposal is based on the following reasoning. As discussed above, all PTS proteins, including EIII<sup>Glc</sup>, can be phosphorylated and dephosphorylated. The phosphorylation level of EIII<sup>Glc</sup> depends on the influx of phosphoryl groups through PEP, EI and HPr and on the

efflux *via* EII<sup>Glc</sup> to the substrate phosphate. Thus in mutants defective in the general proteins EI and HPr, EIII<sup>Glc</sup> will be in the non-phosphorylated form. This is also the predominant form of EIII<sup>Glc</sup> when wild type cells are growing in the presence of a PTS carbohydrate such as glucose. Thus, inhibition of non-PTS uptake systems by non-phosphorylated EIII<sup>Glc</sup> will occur in *ptsH* and *ptsI* mutants and in wild type strains growing in the presence of a PTS carbohydrate, and this inhibition will be relieved by *crr* mutations since the inhibitor, non-phosphorylated EIII<sup>Glc</sup>, is eliminated. In addition, it has been shown by *in vitro* experiments that purified EIII<sup>Glc</sup> can bind to the lactose permease (Osumi and Saier, 1982).

In the second process postulated to involve EIII<sup>Glc</sup>-mediated regulation, phospho-EIII<sup>Glc</sup> is thought to be an activator of adenylate cyclase (Fig. 1.3). Adenylate cyclase is involved in the synthesis of cAMP which is required for the expression of many catabolic operons. Thus, phospho-EIII<sup>Glc</sup> regulates the transcription of catabolic operons and the genes encoding transport systems by modulating the level of intracellular cAMP and CAP. While the involvement of EIII<sup>Glc</sup> in adenylate cyclase activation is established, it appears that EIII<sup>Glc</sup> may not be the only component of the PTS involved in adenylate cyclase activation. Recent evidence suggests that the combined action of HPr, EI and EIII<sup>Glc</sup> activates adenylate cyclase (Levy *et al.*, 1990).

**HPr-MEDIATED REGULATION:** HPr can be phosphorylated in two different ways in Gram-positive bacteria. During carbohydrate uptake a phosphoryl group is transferred from phospho-EI to a histidine residue of HPr. HPr may also be phosphorylated by an ATP-dependent protein kinase (HPr kinase) which transfers a phosphoryl group to a serine residue (Ser46) (Reizer *et al.*, 1988; Reizer, 1989; Deutscher *et al.*, 1989). The phosphoryl group can be removed by a phosphoprotein phosphatase. Since the activities of the HPr kinase and the phosphoprotein phosphatase appear to be regulated by the concentration of various cellular metabolites, including fructose 1,6-bisphosphate and inorganic phosphate, and since the phospho-Ser-HPr is less active in catalyzing the transfer of the phosphoryl group from phospho-EI to the various EII and EIII proteins, the activity of the PTS can be regulated by intracellular metabolites. In addition, the expulsion

of certain solutes from Gram-positive bacteria by PTS carbohydrates like glucose is also thought to be connected to the phosphorylation of the serine residue of HPr. The latter process is proposed to involve a sugar-6-phosphate phosphatase kinase, however, the mechanism is unknown. This type of regulation, phosphorylation of HPr on a serine residue, is found only in Gram-positive bacteria.

#### 1.4 SUCROSE UTILIZATION SYSTEMS IN BACTERIA

The sucrose utilization system of the Gram-positive bacterium *B. subtilis* has been the subject of extensive research (Lepesant *et al.*, 1972; Lepesant *et al.*, 1976; Klier and Rapoport, 1988). In the last decade, a series of elegant experiments has allowed an understanding of the molecular mechanisms regulating sucrose metabolism in *B. subtilis* (Debarbouille *et al.*, 1990). Sucrose metabolism by species of the Gram-positive genus *Streptococcus* has been shown to play an important role in the ability of these organisms to initiate carious lesions in teeth (Loesche, 1986). In this regard, the sucrose utilization system of *S. mutans* has been extensively researched (Sato *et al.*, 1989). The only sucrose utilization systems from Gram-negative bacteria which have been studied extensively are the plasmid-encoded pUR400 system from *S. typhimurium* and the chromosomally encoded system from *K. pneumoniae* (Ebner and Lengeler, 1988; Schmid *et al.*, 1988; Sprenger and Lengeler, 1988). In all these bacteria, sucrose metabolism has been shown to involve sucrose-specific transport by an EII<sup>Scr</sup> of the PEP-dependent PTS. Since sucrose metabolism has been studied mainly in *B. subtilis*, *S. mutans*, *S. typhimurium* and *K. pneumoniae*, these bacteria will be the focus of this review. However, it should be noted that this discussion is by no means exhaustive and that sucrose metabolism has been studied to some extent in numerous other bacteria. In addition, the PTS does not appear to be involved in sucrose metabolism in certain bacteria such as *Zymomonas mobilis* (Preziosi *et al.*, 1990) and *Bacteroides fragilis* (Scholle *et al.*, 1990). Finally, the current understanding of sucrose metabolism in *V. alginolyticus* will be discussed thereby enabling the aims of this thesis to be outlined.

### 1.4.1 *B. subtilis*

The pathway of sucrose metabolism in *B. subtilis* has been studied extensively (Lepesant *et al.*, 1972; Lepesant *et al.*, 1976) (Fig. 1.4). Two saccharolytic enzymes, sucrase (gene *sacA*) and levansucrase (gene *sacB*), can be detected in crude extracts of *B. subtilis* 168 after induction by sucrose. Sucrase is an intracellular enzyme, whereas levansucrase is secreted. Both enzymes act as  $\beta$ -D-fructofuranoside fructohydrolases and are therefore able to hydrolyze sucrose. Levansucrase, in addition, catalyzes the formation of the high-molecular weight fructose polymer levan. A third structural gene, *sacP*, codes for an inducible sucrose transport protein (EII<sup>Scr</sup>) of the PTS.

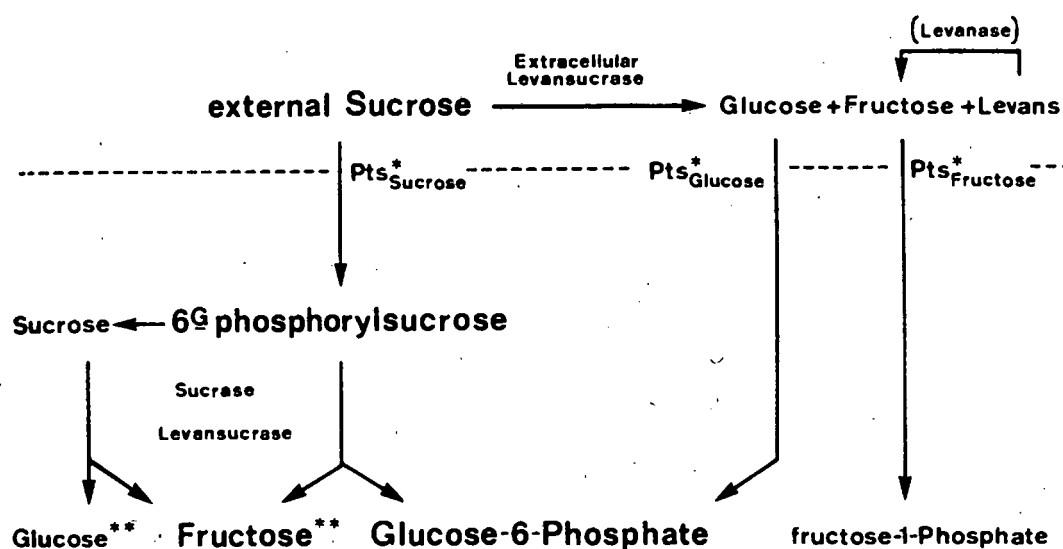
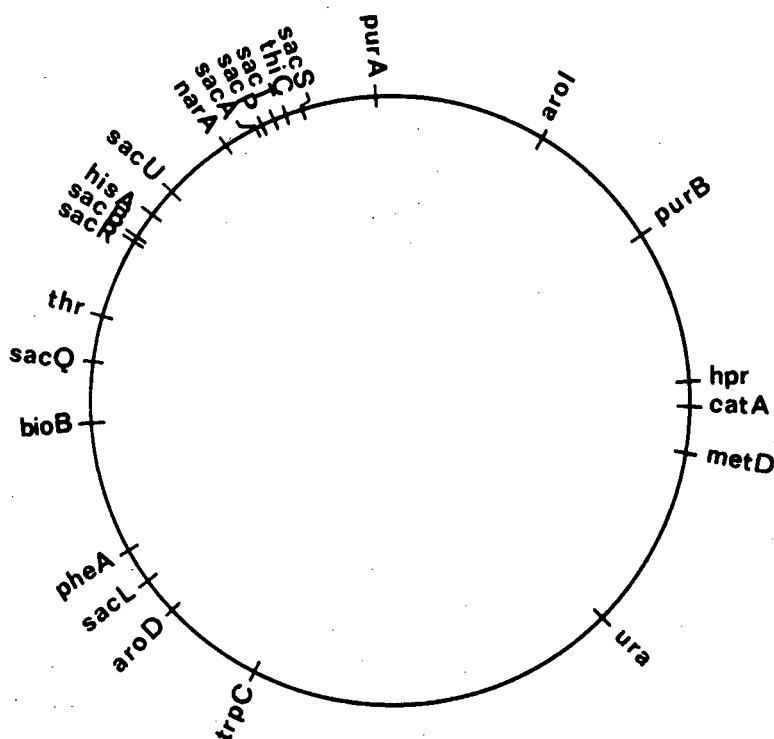


FIG. 1.4 Pathway of sucrose metabolism in *B. subtilis*. One asterisk indicates the reactions catalyzed by the PTS. Two asterisks indicate the subsequent metabolism of intracellular glucose and fructose which involves phosphorylation by specific kinases or, in the case of fructose, exit followed by phosphorylation by the fructose PTS (after Lepesant *et al.*, 1976).

Another saccharolytic enzyme, levanase (gene *sacC*), has been characterized. This enzyme cannot be detected in the wild type strain of *B. subtilis*, and its presence was only revealed after the isolation of a class of mutants referred to as *sacL* (Kunst *et al.*, 1977). Levanase is an extracellular enzyme that, in addition to being a  $\beta$ -D-fructofuranoside fructohydrolase, can hydrolyze both levan and inulin, leading to the production of free fructose. Sucrase has been purified and has a molecular

weight of 55 000 (Kunst *et al.*, 1974a), whereas levansucrase, whose low-resolution crystallographic structure has been determined (LeBrun and van Rapenbusch, 1980), has a molecular weight of 50 000 (Gay *et al.*, 1983; Steinmetz *et al.*, 1985).

**GENETIC LOCI:** The *sacA* and *sacP* genes are closely linked on the *B. subtilis* chromosome while the *sacB* gene maps at a different position (Lepesant *et al.*, 1974a; Lepesant *et al.*, 1974b) (Fig. 1.5). The characterization of several classes of regulatory mutations that affected levansucrase, sucrase and sucrose transport protein synthesis led to the identification of 3 regulatory loci, *sacR*, *sacT* and *sacS* (Lepesant *et al.*, 1972; Lepesant *et al.*, 1976).

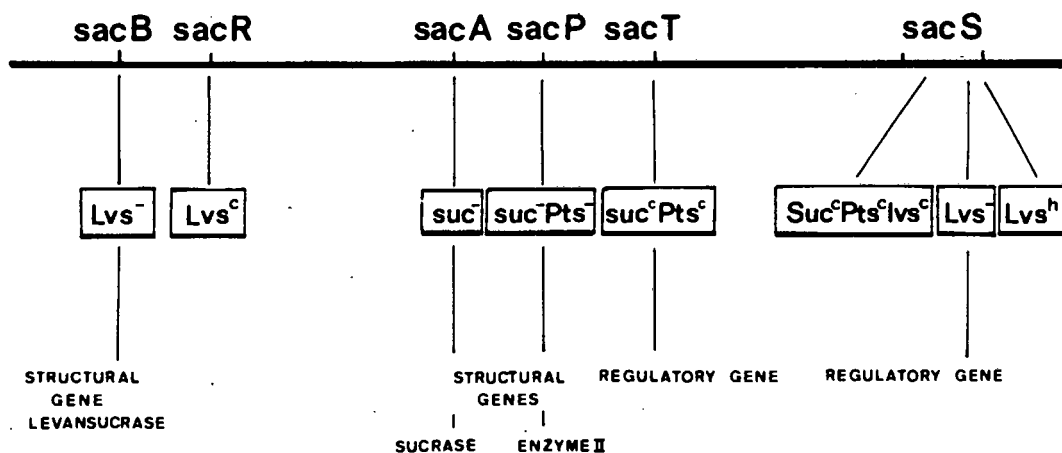


**FIG. 1.5** Simplified genetic map of the *B. subtilis* chromosome (after Lepesant *et al.*, 1976).

The *sacR* locus was originally defined by a series of constitutive mutations (*sacR<sup>c</sup>*) (Lepesant *et al.*, 1972). Levansucrase was synthesized constitutively in the *sacR<sup>c</sup>* mutants (*Lvs<sup>c</sup>* phenotype), whereas sucrose transport and sucrase remained normally inducible in those strains (Fig. 1.6). The *sacR* locus was tightly linked to the *sacB* gene (Fig. 1.5). The *sacT* locus was defined by a single constitutive

mutation (*sacT<sup>c</sup>*) that mapped close to the *sacA* and *sacP* genes (Lepesant *et al.*, 1974a) (Fig. 1.5). Sucrose transport and sucrase activities were synthesized constitutively in strains carrying *sacT<sup>c</sup>* (*Pts<sup>c</sup>* and *Suc<sup>c</sup>* phenotype), and levansucrase remained inducible by sucrose (Fig. 1.6). Recently, nt sequencing of the *sacT* locus revealed that this region contained a gene named *sacT* (Debarbouille *et al.*, 1990).

**A**



**B**

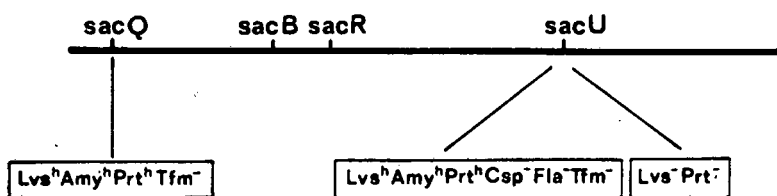


FIG. 1.6 Summary of the phenotypes of the mutations defining the structural genes and regulatory loci/genes of the *B. subtilis* sucrose utilization system (A) and the *sacU* (now *degU*) and *sacQ* (now *degQ*) genes (B). Abbreviations: *Lvs*, levansucrase; *Suc*, sucrase; *Prt*, extracellular serine and metal proteases; *Amy*,  $\alpha$ -amylase; *Fla*, flagella; *Tfm*, competence for transformation; *Csp*, control of sporulation; *Pts*, PEP-dependent sucrose phosphotransferase system; superscript minus, deficiency; superscript c, constitutivity; superscript h, hyperproduction (after Lepesant *et al.*, 1976).

The *sacS* locus affected the synthesis of the sucrose transport system, sucrase and levansucrase (Lepesant *et al.*, 1972). This locus was linked to the *sacA-sacP-sacT* cluster by generalized transduction, but it was completely separate from this group (Fig. 1.5). Three types of mutations have been defined by this locus. *SacS<sup>c</sup>* mutations led to a constitutive phenotype of sucrose transport, sucrase and levansucrase (*Pts<sup>c</sup>*, *Suc<sup>c</sup>*, *Lvs<sup>c</sup>* phenotypes), whereas *sacS<sup>-</sup>* and *sacS<sup>h</sup>* mutations affected only levansucrase production, leading to insignificant (*Lvs<sup>-</sup>* phenotype) and high-level synthesis (*Lvs<sup>h</sup>* phenotype), respectively (Fig. 1.6). Recently, nt sequencing of the *sacS* locus revealed that this region contained 2 genes designated *sacX* and *sacY* (Zukowski *et al.*, 1990).

The systematic isolation of mutations affected in levansucrase synthesis led to the identification of two other separate loci, *sacQ* and *sacU* (Kunst *et al.*, 1974b) (Fig. 1.5 and Fig. 1.6). The *sacU* locus was originally defined by two series of mutants referred to as *sacU<sup>h</sup>* and *sacU<sup>-</sup>*, which exhibited respectively a hyperproduction (*Lvs<sup>h</sup>* phenotype) and a hypoproduction (*Lvs<sup>-</sup>* phenotype) of levansucrase. So far a single *sacQ* mutant has been isolated which displayed a *Lvs<sup>h</sup>* phenotype. Interestingly, the strains carrying the *sacU* and *sacQ* mutations displayed extended pleiotropy indicating that these two loci had a regulatory role in a physiological process of general importance (Fig. 1.6). Extensive research into these pleiotropic effects has shown that the *sacQ* and *sacU* loci contain genes, initially called *sacQ* and *sacU* (Yang *et al.*, 1986; Kunst *et al.*, 1988), but apparently renamed *degQ* and *degU*, respectively (Msadek *et al.*, 1990). The *degS*, *degU*, *degQ* and *degR* genes have been shown to encode proteins involved in a signal transduction pathway controlling the rates of synthesis of degradative enzymes, including an intracellular protease and several secreted enzymes: levansucrase, proteases,  $\alpha$ -amylase,  $\beta$ -glucanase(s) and xylanase (Msadek *et al.*, 1990). This pathway has also been shown to affect transformation by exogenous DNA, presence of flagella and sporulation efficiency in the presence of glucose (Kunst *et al.*, 1974b; Msadek *et al.*, 1990).

**SUCRASE AND EII<sup>S<sub>cr</sub></sup>:** The *sacA* and *sacP* genes encoding the *B. subtilis* sucrase and EII<sup>S<sub>cr</sub></sup> protein, respectively, have been cloned and expressed in *E. coli* (Fouet *et al.*, 1982; Fouet *et al.*, 1986; Fouet *et al.*, 1987). The *sacA* gene has been

sequenced and the deduced aa sequence of the *sacA* gene product gives a protein with  $M_r$  54 827, which is consistent with that of the purified sucrase (Fouet *et al.*, 1986). The aa sequence of the sucrase revealed no significant homology with the aa sequence of the *B. subtilis* levansucrase, suggesting that the 2 saccharolytic enzymes from *B. subtilis* were not derived from a common ancestral gene. Strong homology was detected, however, between the *B. subtilis* sucrase and yeast invertase (*SUC2* gene product). The *sacP* gene has been sequenced and the deduced aa sequence of the *sacP* gene product gives a protein with  $M_r$  of 48 945 (Fouet *et al.*, 1987). *E. coli* cells transformed with the *B. subtilis sacP* gene transported and phosphorylated sucrose indicating that the *sacP* gene product was an EII<sup>Scr</sup> of the sucrose PTS of *B. subtilis*. *E. coli* cells harbouring *sacA* and *sacP* genes grew well in medium containing sucrose as the sole carbon source, demonstrating that the EII<sup>Scr</sup> interacted with the other nonspecific components of the *E. coli* PTS. The aa sequence of the *B. subtilis* EII<sup>Scr</sup> was compared to that of 3 other EII proteins from enteric bacteria: EII<sup>Mtl</sup> from *S. typhimurium* and EII<sup>Glc</sup> and EII<sup>Bgl</sup> from *E. coli* (Fouet *et al.*, 1987). When EII<sup>Scr</sup> and EII<sup>Glc</sup> were compared, the C-terminal part of EII<sup>Glc</sup> was found to share similarity with the N-terminal part of EII<sup>Scr</sup> (corresponds to the hydrophilic EIII-binding domain discussed in section 1.3.1), and a weaker similarity was found for the remaining parts of the sequences. The greatest similarity was found between EII<sup>Scr</sup> and EII<sup>Bgl</sup> which showed strong similarity in their N-terminal regions. Comparison of the four EII aa sequences revealed several blocks of similarity along the sequences consistent with the domains discussed previously (section 1.3.1), and reflecting the common properties of these carbohydrate-specific membrane proteins of the PTS. The nucleotide sequences encoding EII<sup>Scr</sup> and sucrase overlap at the sequence ATGA, where ATG is the initiation codon of the *sacA* gene and TGA is the stop codon of the *sacP* gene. Thus it was proposed that *sacP* and *sacA* were part of an operon in which *sacA* was distal to *sacP* (Fouet *et al.*, 1987).

**LEVANASE:** The *sacC* gene encoding the *B. subtilis* levanase has been cloned and expressed in *E. coli* (Martin *et al.*, 1987a). The cloned gene was mapped near the *sacL* locus on the *B. subtilis* chromosome by generalized transduction. Production of the enzyme was demonstrated both in *B. subtilis* and *E. coli*. The presence of the

*sacC* gene allowed *E. coli* to grow on sucrose as the sole source of carbon. The complete nt sequence of the *sacC* gene has been determined and encodes a protein of  $M_r$  75 866, including a putative signal peptide similar to known *Bacillus* signal sequences. The aa sequence of levanase was compared to that of the other saccharolytic enzymes of *B. subtilis* and to yeast invertase, and found to be homologous to sucrase and yeast invertase but not to levansucrase. A cysteine residue has been implicated in the active site of levanase, sucrase and invertase. It appears that expression of the *sacC* gene is not regulated by *sacR*, *sacS*, *sacT*, *degU* and *degQ* regulatory loci/genes. Expression of the *sacC* gene was studied by using transcriptional and translational fusions (Martin *et al.*, 1989). It was shown that the degradative products of levan or inulin and low concentrations of fructose were able to induce *sacC* expression. In the wild type strain and in a constitutive overproducing *sacL* mutant, levanase synthesis was repressed by glucose and fructose. The *sacC* gene appeared to be the distal gene of an operon transcribed from a fructose-inducible promoter 2.7 kb upstream from the *sacC* gene. Two constitutive *sacL* mutations were mapped in the promoter-proximal region of this operon.

**LEVANSUCRASE:** The *sacB* gene encoding the *B. subtilis* levansucrase and its *sacR* control region were cloned by 2 groups of workers (Gay *et al.*, 1983; Fouet *et al.*, 1984) and the nt sequence of the *sacB* gene and its flanking regions determined (Fouet *et al.*, 1984; Steinmetz *et al.*, 1985). In contrast to the results obtained by Fouet *et al.* (1984), Gay *et al.* (1983) detected levansucrase activity only in crude extracts prepared from exponential-phase *E. coli* transformants. Fouet *et al.* (1984), on the other hand, detected levansucrase activity in the extracellular supernatant fluid of transformed *E. coli* minicells containing the recombinant *sacB*-carrying plasmid. Secretion of levansucrase was shown to be specific for this minicell-producing *E. coli* strain, and could therefore have been ascribed to its membrane defects. The possibility of protein leakage was however, ruled out, since no concomitant  $\beta$ -lactamase activity was detected in the supernatant fluid. The existence of the precursor form of levansucrase was demonstrated in the minicell-producing *E. coli* strain, and had an apparent  $M_r$  of 53 000, which was reduced to a  $M_r$  of 50 000 after processing and translocation. The precursor

levansucrase protein sequence, deduced from the nt sequence of the *sacB* gene, is a 473-aa polypeptide, which includes a 444-aa sequence almost identical to that already determined for the mature levansucrase. The 29 aa located upstream of the mature sequence are typical of a signal sequence with a hydrophilic region followed by a hydrophobic stretch with an alanine residue at position -1.

**REGULATION:** In *B. subtilis* the regulation of *sacB* expression involves primarily the *sacR* and *sacS* loci while the regulation of the *sacPA* operon appears to involve primarily the *sacT* locus. Interestingly, as will be discussed, there appears to be a degree of "cross-talk" between these two regulatory systems.

The *sacR* locus is a 400-bp regulatory region which contains the *sacB* promoter and the targets of the products of *degU*, *degQ*, and *sacS* (Aymerich *et al.*, 1986; Shimotsu and Henner, 1986; Zukowski and Miller, 1986; Klier *et al.*, 1987). S1 nuclease mapping of the *sacB* promoter defined the transcription start site 199 bp upstream of the *sacB* coding sequence (Shimotsu and Henner, 1986). The products of the *degU* and *degQ* genes were shown to activate transcription initiation from the *sacB* promoter (Aymerich *et al.*, 1986; Zukowski and Miller, 1986; Klier *et al.*, 1987). Fusions between the *sacB* promoter and heterologous genes were constructed and gave constitutive expression of these genes. The levels of expression were increased by the presence of the *degU<sup>h</sup>* and *degQ<sup>h</sup>* alleles. Moreover, strains carrying these alleles exhibited increased steady state levels of transcripts from the *sacB* promoter. The target of the products of the *degU* and *degQ* genes was shown to be upstream of the *sacB* promoter, but no direct interaction was shown. The region between the promoter and *sacB* coding sequence consists of a short open reading frame (not translated *in vivo*) which contains a region of dyad symmetry shown to act as a transcriptional terminator structure (Shimotsu and Henner, 1986). This transcriptional terminator was shown to be involved in *sacB* induction by sucrose just downstream of the promoter. Mutations (such as the *sacR<sup>c</sup>* mutations) or deletions altering this structure made *sacB* expression constitutive. It was shown that the *sacB* promoter was constitutive but that transcripts stopped at the terminator in the absence of sucrose and extended past the terminator only in the presence of sucrose. Therefore it was proposed that a positive regulatory protein,

activated by sucrose, binds at or near the stem region of the terminator structure of the nascent transcript, preventing formation of the terminator and allowing transcription of the *sacB* gene. A candidate for such a positive regulatory protein, which would act as an antiterminator protein, was suggested to be a product of the *sacS* locus (Shimotsu and Henner, 1986). When the *sacB* gene was fused to a heterologous gene and the fusion introduced into a *B. subtilis* strain with a *sacS<sup>c</sup>* mutation, the resulting strain showed constitutive expression of the fusion, supporting the proposal that a product of the *sacS* locus was a *trans* acting positive regulatory protein (Shimotsu and Henner, 1986). This result was supported by results obtained with the *sacS<sup>c</sup>* allele cloned on a multicopy plasmid (Debarbouille *et al.*, 1987). It was shown that levansucrase constitutivity conferred by the *sacS<sup>c</sup>*-containing plasmid was dominant over the wild type phenotype (inducible levansucrase synthesis), when *sacS<sup>c</sup>* was present in multiple copies.

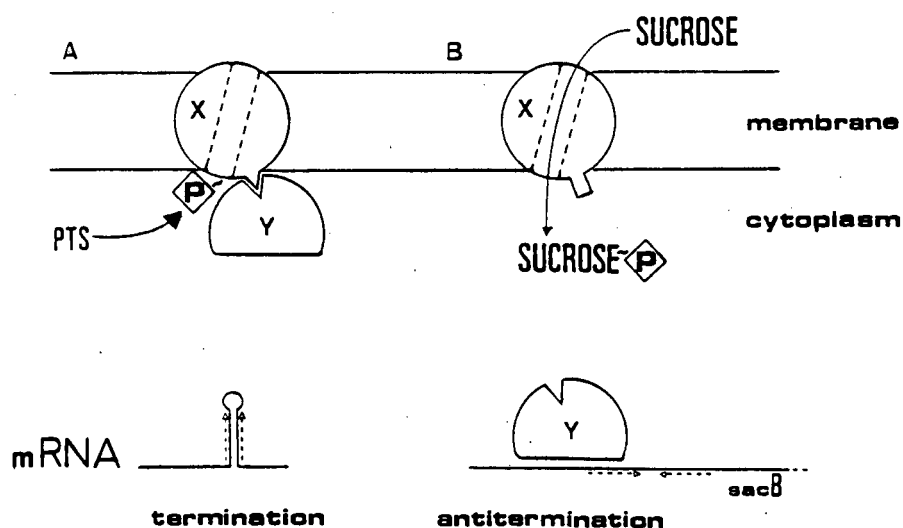
Genetic and sequence analyses have shown that the *sacS* locus of *B. subtilis* is an operon which consists of 2 genes, *sacX* and *sacY* (Aymerich and Steinmetz, 1987; Steinmetz *et al.*, 1989; Crutz *et al.*, 1990; Zukowski *et al.*, 1990). The *sacY* gene encodes the positive regulatory protein (280 aa;  $M_r$  32 465) discussed above while the *sacX* gene encodes a negative regulatory protein (459 aa;  $M_r$  49 024). The mutations in the *sacS* locus causing levansucrase deficiency were found to be most likely *sacY* null mutations. In addition, a frame shift mutation in *sacY* or a deletion removing part of *sacX* and all of *sacY* both resulted in levansucrase deficiency. Such mutations or deletions causing levansucrase deficiency were suppressed by mutations which destroyed the terminator structure in the *sacR* locus. These results together suggested that the *sacY* gene encoded a positive regulatory protein which would act as an antiterminator (Crutz *et al.* 1990). This conclusion was supported by the strong aa sequence similarities found between the *sacY* gene product and the *E. coli bglG* (formerly *bglC*) gene product, which has been shown to be a positive regulator of the *bgl* operon and to act as an antiterminator (Schnetz and Rak, 1988; Schnetz and Rak, 1990; Zukowski *et al.*, 1990).

The *sacX* gene of *B. subtilis* was detected upstream from the *sacY* gene in the *sacS* locus after insertion of the transposon Tn917-*lac* (Aymerich and Steinmetz, 1987). The strains harbouring the transposon showed constitutive *sacB* expression. Similarly it was shown that a deletion in *sacX* resulted in constitutive *sacB* expression (Crutz *et al.*, 1990). However, as already mentioned, a frameshift mutation in *sacY* and a deletion removing part of *sacX* and all of *sacY* both caused levansucrase deficiency. Thus a *sacY* null mutation was epistatic on a *sacX* deletion, suggesting that the *sacX* gene product exerted its control on *sacB* via a negative control on the *sacY* gene or its product (Crutz *et al.*, 1990). This inhibition was observed even when *sacY* was expressed from a heterologous promoter, suggesting that the *sacX* gene product inhibited the activity of the *sacY* gene product. Interestingly, the deduced aa sequence of the *sacX* gene product was found to share 56%, 37% and 47% identity with the EIIS<sup>Scr</sup> proteins from *B. subtilis*, *S. mutans* and *S. typhimurium* (pUR400 system), respectively. This suggested that the *sacX* gene product was a *bona fide* member of the EIIS<sup>Scr</sup> family. Since *sacP* mutants have been found not to transport sucrose at a detectable level, it was suggested that the *sacX* gene product was an inefficient EIIS<sup>Scr</sup>, or, alternatively that it was poorly expressed (Crutz *et al.*, 1990).

*B. subtilis ptsI* mutants have been found to synthesize levansucrase constitutively (Gonzy-Treboul and Steinmetz, 1987). This phenotype was also conferred by a large deletion in the *pts* operon (Crutz *et al.*, 1990). This constitutive levansucrase activity was abolished in double mutants carrying a *sacY* null mutation. This suggested that EI caused *sacB* repression via a negative effect on the *sacY* gene or its product. The *sacY* gene was fused to a heterologous gene and transcription of the fusion was studied in strains of *B. subtilis* carrying a deletion of the *pts* operon. The deletion in the *pts* operon did not derepress transcription of the fusion, indicating that the negative effect of the PTS in *sacB* induction was likely via an inhibition of the function of the *sacY* gene product.

Based on these and other results, it was proposed that EI of the PTS regulated *sacB* induction by inhibiting the *sacY* gene product (SacY) via a regulatory

phosphorylation cascade involving the *sacX* gene product (SacX) (Crutz *et al.*, 1990) (Fig. 1.7). SacX might be phosphorylated by EI, probably *via* HPr and EIII. Phosphorylated SacX would inhibit the antiterminator SacY. Thus termination would occur at the terminator structure in the *sacR* locus, and *sacB* would not be transcribed. In the presence of sucrose, SacX would transport and phosphorylate this sugar. This would dephosphorylate SacX and consequently relieve the inhibition of SacY, which would then prevent termination and allow transcription of *sacB* (Fig. 1.7). A similar model has been proposed for the *E. coli bgl* operon (Schnetzer and Rak, 1988; Schnetz and Rak, 1990).



**FIG. 1.7** Model for the control of *sacB* induction by the PTS. Here, the PTS designates the cytoplasmic PTS cascade (EI, HPr and probably EIII). The SacX protein (X) is phosphorylated by this cascade. In this state it inhibits the SacY antiterminator (Y); for example, by sequestering it, as shown in the figure or by phosphorylating it. This prevents antitermination and transcription of the *sacB* gene (A). The presence of external sucrose (B) results in its transport and concomitant phosphorylation by SacX. The resulting dephosphorylation of SacX relieves SacY inhibition, permitting antitermination at *sacR* and transcription of the *sacB* gene (after Crutz *et al.*, 1990).

Since certain mutations in the *sacS* locus rendered expression of the *sacA* gene constitutive, it has been suggested that the *sacS* locus played a central role in induction of the *sacA* gene in addition to induction of the *sacB* gene. Other observations, however, seemed to contradict this view; higher inducer concentration was required to obtain full induction of *sacB*, and sucrose analogues were good

inducers of *sacA* but not of *sacB* (Lepesant *et al.*, 1976). Recently it was found that a *sacS* deletion removing all of *sacY* and part of *sacX* did not abolish induction of either the *sacA* gene or the *sacB* gene (measured using a fusion of *sacR* to a heterologous reporter gene) (Steinmetz *et al.*, 1989). Under certain growth conditions the expression of the *sacA* gene was weakly affected by the deletion, and almost normal *sacY*-independent induction of the *sacA* gene was observed. Under the same growth conditions the expression of the *sacB* gene was strongly decreased and the *sacY*-independent induction of the *sacB* gene was low but reproducibly observed in both *degU*<sup>+</sup> and *degU*<sup>h</sup> backgrounds. Thus, it was proposed that a *sacY*-independent mechanism allowed both *sacA* and *sacB* induction in the deletion mutant under certain growth conditions, but that this mechanism was inefficient for *sacB*.

This second *sacY*-independent mechanism has been shown to involve the *sacT* locus (Steinmetz *et al.*, 1989). Previously it was found that the *sacT*<sup>c</sup> mutation rendered *sacA* and *sacP* expression constitutive while *sacB* expression remained inducible by sucrose (Lepesant *et al.*, 1976). Therefore, it was suggested that the *sacT* locus was a *cis*-regulatory region of the *sacP-sacA* operon. Reexamination of the phenotype of the *sacT*<sup>c</sup> mutant revealed that this mutation, contrary to the initial observation, caused expression of the *sacB* gene to be semiconstitutive but still overinducible by sucrose in a *sacT*<sup>c</sup> *degU*<sup>h</sup> double mutant (Steinmetz *et al.*, 1989). In addition, this semiconstitutive phenotype was *sacY*-independent since it was observed for a *B. subtilis* strain containing both the *sacT*<sup>c</sup> mutation and a deletion of *sacY* and part of *sacX*. On the basis of these results it was proposed that the *sacT* locus was not necessarily purely a *cis*-regulatory region and that it contained a gene encoding a diffusible regulator primarily involved in *sacA* induction but also able to regulate *sacB* expression. Cloning and nt sequencing of the *sacT* locus have provided strong support for this hypothesis (Debarbouille *et al.*, 1990). Nucleotide sequencing and analysis of the region immediately upstream of the *sacPA* operon indicated that the *sacT*<sup>c</sup> mutation originally defining the *sacT* locus resided in a gene now referred to as the *sacT* gene. The *sacT* gene product was shown to be involved in *sacPA* operon expression since a *B. subtilis* strain deleted for the *sacT* gene showed a 10-fold decrease in sucrase synthesis as compared to the wild type strain. As already

mentioned, residual *sacB* induction was observed in a *B. subtilis* strain deleted for *sacX* and *sacY* and most likely involved the *sacT* gene product. A reciprocal phenomenon was observed for the *B. subtilis* strain deleted for the *sacT* gene; low level expression of *sacPA* was induced by sucrose, suggesting the involvement of the *sacY* gene product. The deduced aa sequence of the *sacT* gene product (276 aa;  $M_r$  32 037) was shown to be significantly similar to those of the *sacY* and the *bglG* gene products, with 48% and 35% of the aa residues being identical, respectively. An intergenic region of 870 bp was found between the end of the *sacT* gene and the putative promoter region of the *sacPA* operon. This promoter region was located within the 245 bp preceding the initiation codon of *sacP*. A region of dyad symmetry is present 70 bp upstream of the initiation codon of *sacP*. This structure was shown to be similar to the *sacB* transcriptional terminator. Thus, it was speculated that the *sacT* gene encoded an antiterminator interacting directly or indirectly with the palindromic structure upstream of *sacPA*. It was shown that the *sacPA* operon was constitutively expressed in a *B. subtilis ptsI* mutant and in a strain deleted for the *pts* operon. This indicated that the PTS was involved in the induction of the *sacPA* operon. However, this PTS-mediated regulation most likely did not involve the *sacX* gene product since the *sacPA* operon was still inducible in a *B. subtilis* strain deleted for *sacX* and *sacY*. Therefore it was proposed that by analogy with the *sacX sacY* system, the *sacT* gene product would be regulated by the *sacP* gene product (EII<sup>Scr</sup>) or by another EII-like regulator.

Therefore, it appears that *B. subtilis* has two systems that allow induction of saccharolytic enzymes: (i) a *sacY*-dependent system, efficient for *sacB* induction (involving the *sacY* gene product as an antiterminator) but also involved in *sacPA* induction albeit inefficiently, and (ii) a *sacT*-dependent system, efficient for *sacPA* induction (involving the *sacT* gene possibly also as an antiterminator) but also involved in *sacB* induction albeit inefficiently.

A new locus involved in sucrose metabolism in *B. subtilis*, *sacV*, was identified by Martin *et al.* (1987b). The *sacV* locus, limited to a 0.5-kb DNA fragment, was shown to stimulate transcription of the *sacB* gene after sucrose induction when present on a multicopy plasmid in *B. subtilis*. The *sacV* locus was mapped and sequenced and

appeared to encode a 64-aa peptide. The exact mechanism by which the gene product of *sacV* participates in the stimulation of levansucrase synthesis remains to be elucidated.

#### 1.4.2 *S. mutans*

Sucrose metabolism by the oral pathogen *S. mutans* plays at least two roles in the ability of this organism to initiate carious lesions in teeth (Loesche, 1986). Firstly, sucrose is a substrate for extracellular glucosyltransferases and fructosyltransferases that synthesize glucans and fructans, respectively. Glucans and fructans are important in the colonization of *S. mutans* in the oral cavity. Secondly, sucrose is an efficient fermentation substrate for *S. mutans*, leading to excretion of lactic acid, which is responsible in part for the initiation of dental caries.

**GLUCOSYLTRANSFERASES:** Streptococci have at least two different types of glucosyltransferase activities based on the structure of the product, producing glucans with either predominantly  $\alpha 1,3$  or predominantly  $\alpha 1,6$  linkages (Loesche, 1986; Shiroza *et al.*, 1987). Genes encoding glucosyltransferase activities have been isolated by several groups from various *S. mutans* serotypes. Robeson *et al.* (1983) reported the cloning and expression in *E. coli* of a *gtfA* gene encoding a glucosyltransferase from *S. mutans* UAB90. *E. coli* transformants were able to utilize sucrose as the sole source of carbon, due to the synthesis of the heterologous heat-stable glucosyltransferase, which had a  $M_r$  of 55 000. In the *S. mutans* strain and *E. coli* transformants, the *gtfA*-encoded protein appeared to be embedded in the cytoplasmic membrane with the catalytic site orientated toward the exterior. This enzyme acted on sucrose to yield fructose and a product which contained glucose and failed to migrate from the point of application in paper chromatography. It was on the basis of these results that it was concluded that the product was a glucan and that the enzyme was indeed a glucosyltransferase. It was further speculated that the glucan contained  $\alpha 1,3$  linkages and acted as a primer for other glucosyltransferases. Pucci and Macrina (1985) cloned a *gtfA* gene from *S. mutans* LM7 and found by DNA-DNA hybridization studies that this gene was homologous to the *gtfA* gene cloned by Robeson *et al.* (1983). The complete nt

sequence of the *gtfA* gene from *S. mutans* Ingbritt has been determined (Ferretti *et al.*, 1988). Analysis of the deduced aa sequence indicated that this glucosyltransferase enzyme lacked a secretion signal peptide. A search for homologies between this *gtfA* gene and other genes encoding glucosyltransferases failed to reveal any relatedness. Studies with this glucosyltransferase enzyme obtained several results not expected for a glucosyltransferase: the product made from sucrose was not digested by enzymes which attacked  $\alpha$ 1,6 and  $\alpha$ 1,3 linkages, it did not bind to glucan-binding proteins, there was no antigenic cross-reaction with other *S. mutans* glucosyltransferases, and enzyme activity was only observed in the presence of phosphate buffers (Russell *et al.*, 1988). Closer analysis revealed that this glucosyltransferase converted sucrose to fructose and glucose-1-phosphate in a reaction which took place only in the presence of inorganic phosphate. It was concluded that this glucosyltransferase was a sucrose phosphorylase. Since this enzyme still acted as a glucosyltransferase by transferring glucose from sucrose to phosphate, it was suggested that the name of the gene encoding this enzyme remain as *gtfA*. The gene *gtfB* encoding a glucosyltransferase from *S. mutans* GS-5 has been isolated and characterized (Aoki *et al.*, 1986). This glucosyltransferase catalyzed the formation of glucans with primarily  $\alpha$ 1,3 linkages. Localization studies indicated that most of the glucosyltransferase activity produced in *E. coli* clones was associated with the cytoplasmic membrane. The nt sequence of the *gtfB* gene has been determined and encodes a strongly hydrophilic protein with a  $M_r$  of 165 800 (Shiroza *et al.*, 1987). The deduced aa sequence revealed a typical Gram-positive bacterial signal sequence (34 aa) at the N-terminus of the protein and 3.5 direct repeating units (65 aa each) at the C-terminus. It was proposed that if the glucosyltransferase was cleaved after residue 34, a processed protein of  $M_r$  162 300 would be produced. This was slightly larger than the apparent  $M_r$  of approximately 155 000 determined for the glucosyltransferase after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Aoki *et al.*, 1986), indicating that additional processing may occur.

**FRUCTOSYLTRANSFERASES:** Streptococci have at least two types of fructosyltransferase activities which differ in the structure of the product and produce either an inulin or a levan. The *ftf* gene encoding an inulin-producing

fructosyltransferase from *S. mutans* GS-5 has been isolated and characterized (Sato and Kuramitsu, 1986). Localization studies indicated that most of fructosyltransferase activity produced in *E. coli* clones was secreted into the periplasmic space. The nucleotide sequence of the *ftf* gene has been determined and the deduced aa sequence indicated that the unprocessed fructosyltransferase would have a  $M_r$  of 87 600 (Shiroza and Kuramitsu, 1988). A typical streptococcal signal sequence (34 aa) was found at the N-terminus of the protein and the predicted  $M_r$  of the mature fructosyltransferase was calculated to be 83 800. This  $M_r$  was consistent with that of the fructosyltransferase activity observed in culture fluids of *S. mutans* GS-5. A comparison of the aa sequence of the fructosyltransferase protein with that of levansucrase from *B. subtilis* indicated that the 2 proteins contained regions of homology. Since both enzymes catalyzed the same basic reaction, it was suggested that these homologous regions were required for enzymatic activity. The *ftf* gene was found to be preceded by a region of dyad symmetry, which by analogy to the region of dyad symmetry found upstream from the *B. subtilis* *sacB* gene, was suggested to be involved in regulation of *ftf* expression. The gene encoding a glucan-binding protein has been isolated from *S. mutans* Ingbritt. This protein showed fructosyltransferase activity when isolated from *S. mutans*, but the cloned gene expressed in *E. coli* showed no fructosyltransferase activity (Russell *et al.*, 1985a).

**TRANSPORT AND METABOLISM OF SUCROSE:** During growth on sucrose, *S. mutans* assimilates only 2 to 5% of sucrose carbon as extracellular glucans and fructans (Loesche, 1986). The majority of sucrose is shuttled directly into the homofermentative glycolytic pathway to lactic acid. Sucrose has been shown to enter *S. mutans* directly by a high-affinity PEP-dependent PTS ( $K_m$  70  $\mu$ M) resulting in the accumulation of intracellular sucrose-6-phosphate (St. Martin and Wittenberger, 1979a; Slee and Tanzer, 1979). The sucrose-6-phosphate was shown to be subsequently cleaved by a sucrose-6-phosphate hydrolase to yield glucose-6-phosphate and fructose (Chassey and Porter, 1979; St. Martin and Wittenberger, 1979b). The latter has been proposed to be phosphorylated by a specific fructokinase that has been identified and characterized in *S. mutans* (St. Martin and Wittenberger, 1979a; Porter *et al.*, 1980). Regulatory studies

indicated that the sucrose-6-phosphate hydrolase was synthesized constitutively, while the sucrose PTS was only induced during growth on sucrose (St. Martin and Wittenberger, 1979b). However, the levels of both the sucrose PTS and the sucrose-6-phosphate hydrolase were repressed when fructose was used as a growth substrate.

The genes encoding sucrose-6-phosphate hydrolases from *S. mutans* have been cloned in *E. coli* by several groups (Russell *et al.*, 1985b; Hayakawa *et al.*, 1986; Lunsford and Macrina, 1986). The sucrose-6-phosphate hydrolase expressed by these genes had a  $M_r$  of approximately 58 000. However, the comparable enzyme purified from *S. mutans* strains had a somewhat smaller  $M_r$  of approximately 48 000 (Kuramitsu, 1973; Tanzer *et al.*, 1973). These results suggested different types of posttranslational modification of the enzyme in *E. coli* and *S. mutans*. To investigate this possibility, the nt sequence of the *scrB* gene encoding the sucrose-6-phosphate hydrolase from *S. mutans* GS-5 was determined (Sato and Kuramitsu, 1988). The deduced aa sequence of the enzyme suggested a  $M_r$  of 51 750, which was similar to that estimated for the enzyme isolated from *S. mutans* ( $M_r$  48 000) but lower than that estimated for the enzyme produced in *E. coli* clones ( $M_r$  58 000). It was suggested that the cloned gene product migrated at a slower-than-normal rate or that the enzyme underwent unusual processing in *E. coli*. In addition, it was suggested that, contrary to the previous suggestions, no extensive posttranslational modification of sucrose-6-phosphate hydrolase occurred in *S. mutans*. The deduced aa sequence of the *S. mutans* sucrose-6-phosphate hydrolase showed significant homology with that of the *B. subtilis* sucrase. In addition, a region of aa homology with the *S. mutans* fructosyltransferase and the *B. subtilis* levansucrase was detected.

Detailed characterization of sucrose transport in *S. mutans* has revealed the presence of 3 different uptake systems for sucrose. A high-affinity ( $K_m$  70  $\mu$ M) PEP-dependent PTS has been extensively studied and was shown to involve a sucrose-specific  $EII^{Scr}$  encoded by the *scrA* gene (Sato *et al.*, 1989). A second sucrose PTS with lower affinity for sucrose ( $K_m$  250  $\mu$ M) was discovered in a mutant of *S. mutans* that lacked the high-affinity sucrose PTS (Slee and Tanzer, 1982). Recently, this lower-affinity sucrose PTS was shown to be a high-affinity

trehalose PTS that also recognized sucrose as a substrate (Poy and Jacobson, 1990). A third, low-affinity, sucrose uptake system ( $K_m$  3 mM) which was PTS-independent has also been described (Slee and Tanzer, 1982). While the high-affinity sucrose PTS was shown to be inducible by sucrose, this low-affinity PTS-independent sucrose uptake system was expressed in both glucose and sucrose grown cells. It was proposed that the low-affinity system functioned under conditions of sucrose abundance and rapid growth. The high-affinity sucrose PTS has been shown to be repressed under conditions of sucrose abundance and derepressed when sucrose becomes limiting in the medium (Slee and Tanzer, 1982; Lodge and Jacobson, 1988).

The *scrA* gene encoding the *S. mutans* GS-5 EII<sup>Scr</sup> protein has recently been isolated and characterized (Sato *et al.*, 1989). The cloned fragment containing the *scrA* and *scrB* genes was mapped by transposon mutagenesis and the *scrA* gene found to be located upstream of the *scrB* gene. Nucleotide sequencing confirmed that the *scrA* gene was located immediately upstream of the *scrB* gene and divergently transcribed from the opposite DNA strand. This gene arrangement was compatible with the previous observations that sucrose PTS activity was inducible but sucrose-6-phosphate hydrolase activity was constitutive in *S. mutans* (St. Martin and Wittenberger, 1979b). The *scrA* gene encoded an EII<sup>Scr</sup> protein of 664 aa residues with a calculated  $M_r$  of 69 983, which corresponded well with the  $M_r$  observed for the cloned *scrA* gene product after SDS-PAGE analysis. The hydropathy plot of the EII<sup>Scr</sup> protein revealed that it was relatively hydrophobic containing 51% hydrophobic, 32% hydrophilic, 9% negatively charged and 8% positively charged aa residues. This EII<sup>Scr</sup> protein was larger than the EII<sup>Scr</sup> proteins from *B. subtilis* (460 aa; Fouet *et al.*, 1987) and *S. typhimurium* (pUR400 system) (456 aa; Ebner and Lengeler, 1988). The 491-aa N-terminal sequence of the *S. mutans* EII<sup>Scr</sup> protein showed 34% and 36% identical aa similarity to the EII<sup>Scr</sup> proteins of *B. subtilis* and *S. typhimurium* (pUR400 system), respectively. The 173-aa C-terminus of the *S. mutans* EII<sup>Scr</sup> protein showed 34% identical aa similarity to the *S. typhimurium* EIII<sup>Glc</sup> protein. The *S. mutans* EII<sup>Scr</sup> protein was similar in size to the *E. coli* EII<sup>Bgl</sup> protein (625 aa) and showed 27% identical aa similarity to this protein. Thus, as has been found for the EII<sup>Bgl</sup> and EII<sup>Nag</sup> proteins, the *S. mutans* EII<sup>Scr</sup> protein contained an EIII-like domain suggesting that it was an

EIII-independent EII<sup>Scr</sup> and was clearly distinct from the previously described EII<sup>Scr</sup> proteins (Sato *et al.*, 1989; Lengeler *et al.*, 1990). In addition to the EIII-like domain, the *S. mutans* EII<sup>Scr</sup> appeared to contain the other 2 domains characteristic of EII proteins: an N-terminal hydrophilic EIII-binding domain and a hydrophobic domain (Sato *et al.*, 1989; Lengeler *et al.*, 1990). Partial sequencing of the region downstream of the *scrB* gene revealed the presence of 2 ORF's whose deduced aa sequences shared homology with those of known bacterial regulatory proteins (Sato *et al.*, 1989). This putative regulatory region is currently under investigation.

### 1.4.3 *S. typhimurium* and *K. pneumoniae*

In contrast to the Gram-positive bacteria, comparatively few sucrose utilization systems from Gram-negative bacteria have been studied in detail. Among the *Enterobacteriaceae*, only the tribe *Klebsielleae* is characterized by a stable sucrose-positive phenotype (Lengeler *et al.*, 1982). For the other tribes, including the closely related *Vibrionaceae*, an unstable sucrose phenotype is typical. The genes for such variable metabolic properties normally seem to be located on transmissible elements (plasmids or transposons) of unknown origin. Thus, plasmid free strains of *E. coli* and *S. typhimurium* are unable to take up and ferment sucrose or mutate to a sucrose-positive phenotype, while in epidemiologically related strains of these bacteria this phenotype is common and variably coupled to the presence of plasmids or transposons (Alaeddinoglu and Charles, 1979; Lengeler *et al.*, 1982; Schmid *et al.*, 1982; Doroshenko *et al.*, 1988).

**S. TYPHIMURIUM:** A conjugative plasmid, pUR400, isolated from a clinical sucrose-positive *S. typhimurium* strain enabled *E. coli* transformants to utilize sucrose as the sole source of carbon (Schmid *et al.*, 1982). Sucrose metabolism was found to involve two inducible plasmid-encoded functions. Sucrose was transported by a EII<sup>Scr</sup> protein of the PEP-dependent PTS, and the intracellular sucrose-6-phosphate was hydrolyzed by an intracellular  $\beta$ -D-fructofuranoside fructohydrolase, hereafter called a sucrose-6-phosphate hydrolase. These functions were encoded by *scrA* and *scrB*, respectively, and appeared to be located in an *scr* operon with the

gene order *scr* (O,P) *scrA scrB*. The fact that both genes were found to be simultaneously induced appeared to support this hypothesis. The isolation of a mutation that caused constitutive expression of the *scrA* and *scrB* genes led to the discovery of a third gene, *scrR*, the product of which appeared to exert a negative control on the expression of the *scr* structural genes. Both sucrose transport and sucrose-6-phosphate hydrolase were inducible by fructose, sucrose and raffinose, however, if a *scrB* mutant was used, fructose was the only inducer. This suggested that fructose or a derivative acted as the endogenous inducer. Sucrose transport and sucrose-6-phosphate hydrolase were sensitive to catabolite repression since these two functions were not expressed in an *E. coli* strain deficient in the CAP protein. The active sucrose-6-phosphate hydrolase was shown to be a dimer of  $M_r$  110 000 and to consist of two identical subunits. This enzyme catalyzed the hydrolysis of sucrose-6-phosphate, sucrose and raffinose at an optimal pH of 6.6. The hydrolysis products in the case of sucrose-6-phosphate were fructose and glucose-6-phosphate (Schmid *et al.*, 1982).

The sucrose utilization system from *S. typhimurium* harboured on plasmid pUR400 was subcloned into pBR325 and transformed into *E. coli* (Garcia, 1985). The resulting recombinant *E. coli* were able to utilize sucrose as the sole source of carbon. The results obtained were compatible with the hypothesis proposed by Schmid *et al.* (1982) that the *scrA* and *scrB* genes were located in one operon, however, the gene order appeared to be *scr* (O,P) *scrB scrA* as opposed to the previous order *scr* (O,P) *scrA scrB*.

In an attempt to clarify the discrepancy of the gene order, Schmid *et al.* (1988) reinvestigated the molecular structure of the *S. typhimurium* (pUR400 system) sucrose utilization system. The *scr* genes of the catabolic plasmid pUR400 were subcloned into pBR328 and analyzed in *E. coli* K12. The different genes were mapped by transposon insertion mutagenesis, by restriction endonuclease and deletion mapping, and the corresponding gene products were identified. Besides the known structural genes *scrA*, coding for an EIIS<sup>scr</sup> ( $M_r$  45 000) of the PEP-dependent PTS, and *scrB*, coding for a sucrose-6-phosphate hydrolase ( $M_r$  55 000), two new structural genes were discovered, *scrK* and *scrY*.

Gene *scrK* apparently codes for an intracellular and ATP-dependent fructokinase ( $M_r$  39 000). The physiological role of this fructokinase was the hydrolysis of intracellular fructose derived from the hydrolysis of intracellular sucrose-6-phosphate. The loss of this fructokinase still allowed growth, although at a reduced rate. Gene *scrY* appears to code for an outer membrane sucrose-specific porin ( $M_r$  58 000) required in particular during growth in low sucrose concentrations. Insertional inactivation of *scrY* led to reduced, though not completely negative sucrose fermentation in *E. coli*. The genes were shown to form an *scr* operon and to be arranged in the order *scrK*, *scrY*, *scrA*, *scrB*, *scrR* and transcribed in the direction from *scrK* to *scrR*. Four promoters were tentatively suggested to be located upstream from *scrK*, *scrY*, *scrA* and *scrR*. A fifth promoter immediately adjacent to *scrB* was proposed in order to comply with the results of Garcia (1985). The four genes *scrK*, *scrY*, *scrA* and *scrB* were shown to be under the control of the *scrR* gene product ( $M_r$  37 000). In *scrR*<sup>+</sup> cells, expression of the four structural genes was inducible by sucrose, fructose and fructose-containing oligosaccharides, but was constitutive when *scrR* was deleted.

Since the sucrose transport protein of *S. typhimurium* (pUR400 system) is an EII<sup>Scr</sup> of the PEP-dependent PTS, it requires the cytoplasmic proteins EI and HPr before sucrose transport and concomitant phosphorylation can occur. In addition it was shown that EIII<sup>Glc</sup> played a direct role in the uptake and phosphorylation of sucrose (Lengeler *et al.*, 1982). This indicated that the EII<sup>Scr</sup> protein was EIII<sup>Glc</sup>-dependent and formed an EII<sup>Scr</sup>/EIII<sup>Glc</sup> pair. The nt sequence of the *scrA* gene was recently determined and the deduced aa sequence for the EII<sup>Scr</sup> found to contain 456 aa residues of  $M_r$  45 700 (Ebner and Lengeler, 1988; correction to N-terminal aa sequence in Lengeler *et al.*, 1990). The  $M_r$  for the deduced aa sequence corresponded well with the value of approximately 45 000 observed for the cloned gene product of *scrA* in SDS gel electrophoresis. The EII<sup>Scr</sup> protein contained 58% hydrophobic, 30% hydrophilic, 7% negatively charged and 5% positively charged aa residues and the average hydropathy of the EII<sup>Scr</sup> protein was characteristic of an integral membrane protein. An empirical "best fit" alignment of the aa sequences of 6 EII proteins and an EIII protein was determined: EII<sup>Scr</sup> from *S. typhimurium* (pUR400 system); EII<sup>Scr</sup> from *B. subtilis*; EII<sup>Bgl</sup>, EII<sup>Mtl</sup>, EII<sup>Glc</sup> and EII<sup>Nag</sup> from *E. coli*; and

EIII<sup>Glc</sup> from *S. typhimurium*. At the aa level the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein showed 52% identical aa similarity to the EII<sup>Scr</sup> from *B. subtilis*. Both the latter EII<sup>Scr</sup> proteins showed 27% identical-aa similarity to the N-terminal 455 aa of the *E. coli* EII<sup>Bgl</sup> protein. The C-terminus of the EII<sup>Bgl</sup> protein was shown to have 34% identical aa similarity to the *S. typhimurium* EIII<sup>Glc</sup> protein. The 3 domains characteristic of EII proteins (EIII-binding, hydrophobic and EIII-like; section 1.3.1) were evident when comparing the various EII proteins or EII/EIII pairs. The EII<sup>Scr</sup> of *S. typhimurium* (pUR400 system) contained an N-terminal, hydrophilic EIII-binding domain (aa 1-80) and a hydrophobic domain (aa 101-440). No EIII-like domain was found. This was consistent with the previous results (Lengeler *et al.*, 1982) which indicated that the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein was EIII<sup>Glc</sup>-dependent and interacted with EIII<sup>Glc</sup> during sucrose transport and concomitant phosphorylation.

**K. PNEUMONIAE:** The chromosomally encoded sucrose utilization system from *K. pneumoniae* was found to be very similar to that of *S. typhimurium* (pUR400 system), suggesting a recent common origin of these sucrose utilization systems (Sprenger and Lengeler, 1988). *K. pneumoniae* was found to transport and phosphorylate sucrose by the PEP-dependent PTS. In addition to the 2 soluble and general components EI and HPr of the PTS, a sucrose-specific EII<sup>Scr</sup> (gene *scrA*), together with EIII<sup>Glc</sup> were needed for the vectorial transport of sucrose to generate intracellular sucrose-6-phosphate. This sugar phosphate was found to be hydrolyzed by a hydrolase (invertase, gene *scrB*) to generate glucose-6-phosphate and fructose. The fructose was found to be converted to fructose-6-phosphate by an ATP-dependent fructokinase (gene *scrK*), an enzyme which was shown to be part of the sucrose and not the fructose catabolic pathway. The genes *scrA*, *scrB* and *scrK*, together with a gene *scrR* for a repressor, were found to form a genetic unit located on the chromosome of *K. pneumoniae*. The *scrA* gene encoding the *K. pneumoniae* EII<sup>Scr</sup> protein was recently sequenced (F. Titgemeyer unpublished results as quoted in Lengeler *et al.*, 1990). The aa sequences of the EII<sup>Scr</sup> proteins from *K. pneumoniae*, *S. typhimurium* (pUR400 system), *B. subtilis* and *S. mutans* were used to generate a computer-based hypothetical model of the two-dimensional arrangement of these

proteins in the cytoplasmic membrane (Fig. 1.8). For all 4 EII<sup>Scr</sup> proteins, the general structure and two-thirds of the sequences were highly conserved.

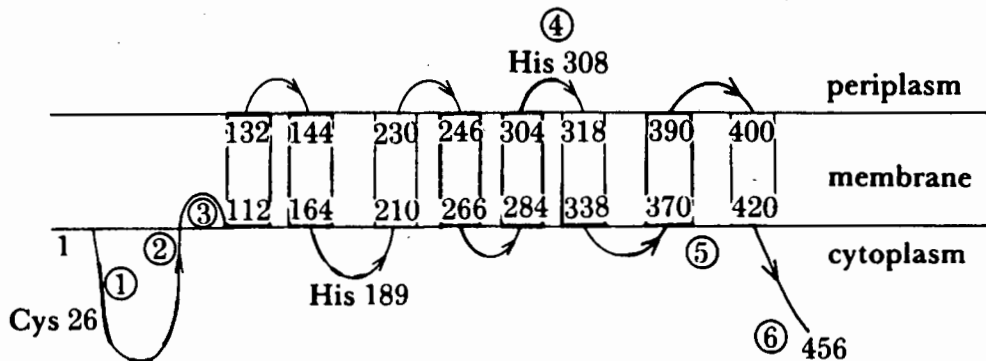


FIG. 1.8 A hypothetical model based on the sequences of the EII<sup>Scr</sup> proteins from *K. pneumoniae*, *S. typhimurium* (pUR400 system), *B. subtilis* and *S. mutans*. The circled numbers designate characteristic structures found in a number of EII proteins and described in detail in section 1.3.1 of the text: 1, highly conserved cysteine residue that may be phosphorylated and pass its phosphoryl group to the substrate; 2 and 6, sequences enriched for charged aa; 3, amphipathic helix; 4, conserved histidine residue; 5, GITE motif. The numbering of aa residues (1-456) is according to the first two sequences. Thin lines indicate non-conserved, thickened lines conserved sequences (after Lengeler *et al.*, 1990).

#### 1.4.4 *V. alginolyticus*

**A MARINE *V. ALGINOLYTICUS* STRAIN:** The sucrose utilization system of a marine *V. alginolyticus* strain has been investigated (Tokuda and Unemoto, 1982; Kakinuma and Unemoto, 1985). It was found for this marine *V. alginolyticus* strain that Na<sup>+</sup> was essential for the uptake of sucrose and could not be replaced by Li<sup>+</sup>, Cs<sup>+</sup> or Rb<sup>+</sup>. Sucrose uptake was completely inhibited by the addition of a proton conductor (CCCP) at acidic pH, but not at alkaline pH, where the primary respiratory-driven Na<sup>+</sup> pump generated the Na<sup>+</sup> electrochemical gradient (see also section 1.2.3). Therefore it was concluded that sucrose transport was driven by the electrochemical potential of Na<sup>+</sup> in this marine *V. alginolyticus* strain. The sucrose was found to accumulate in the cells without concomitant phosphorylation and was subsequently hydrolyzed into glucose and fructose by an intracellular sucrose (Kakinuma and Unemoto, 1985). Synthesis of the sucrose uptake

system was induced simultaneously in *V. alginolyticus*. Sucrase and sucrose uptake activities were low in cells grown on glycerol, glucose, maltose or lactose as carbon sources, but high in cells grown in the presence of sucrose. Addition of glucose to sucrose grown cells repressed the synthesis of sucrose uptake and sucrase activities. These results suggested that sucrose was utilized by a sucrose-inducible, glucose-repressible system in *V. alginolyticus*.

**A COLLAGENOLYTIC *V. ALGINOLYTICUS* STRAIN:** As mentioned previously (section 1.1.2), extensive research has been carried out on a collagenolytic *V. alginolyticus* strain. In addition to the systems mentioned previously, the sucrose utilization system of this collagenolytic *V. alginolyticus* strain has been investigated. The sucrose utilization system of the collagenolytic *V. alginolyticus* strain was found to consist of sucrase and sucrose uptake activities (Scholle *et al.*, 1987). The *V. alginolyticus* sucrose utilization system was cloned on a 10.4-kb DNA fragment (plasmid pVS100; Fig. 1.9) and enabled recombinant *E. coli* cells to utilize sucrose as the sole source of carbon (Scholle *et al.*, 1987).

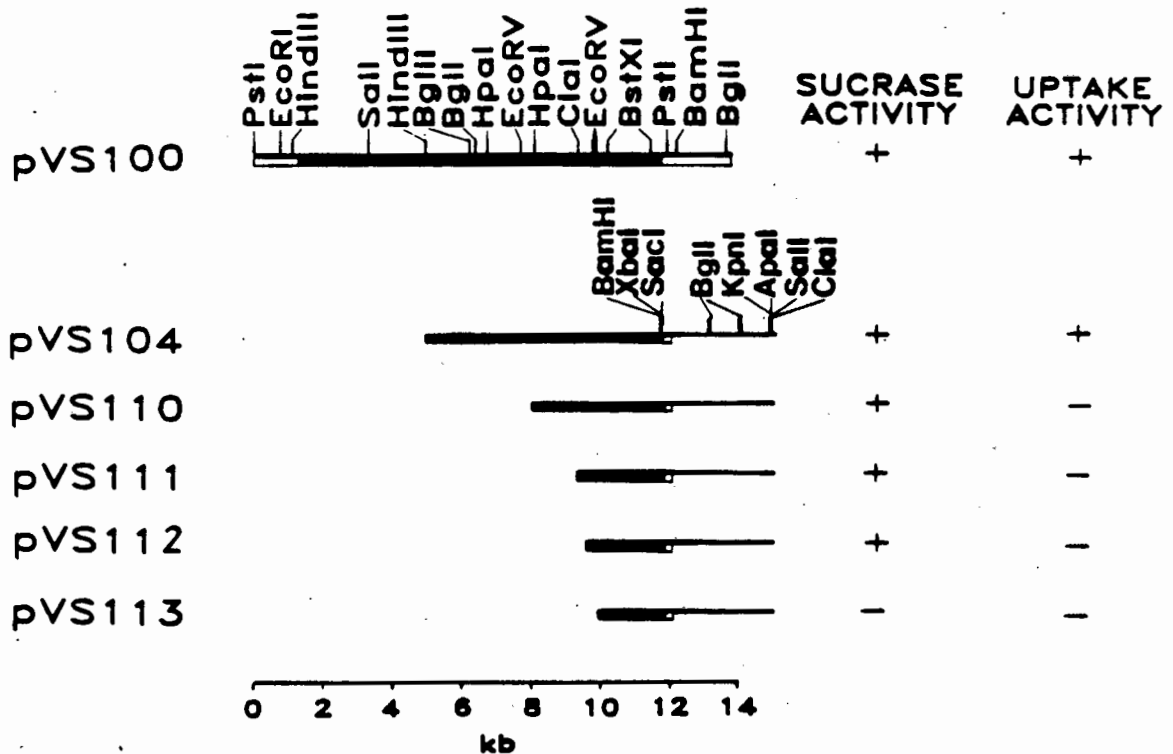


FIG. 1.9 Restriction map, subclones and deletion derivatives of pVS100. The thin lines, and open and closed boxes represent Bluescript vector DNA, pEcoR251 vector DNA and *V. alginolyticus* DNA, respectively. Sucrase and sucrose uptake activities are indicated for each construct (after Scholle *et al.*, 1989).

Progressive deletions from the 5' end of the insert in pVS100 resulted in the loss of sucrose uptake activity, whereas the sucrase activity was retained (Scholle *et al.*, 1989) (Fig. 1.9). Further deletions from the 5' end resulted in the loss of both activities. This indicated that the sucrase-encoding gene was located at the 3' end of the insert and the sucrose uptake-encoding gene(s) was located at the 5' end.

Sucrase activity was detected both intra- and extracellularly in *E. coli* (pVS100) cultures while in *V. alginolyticus* only intracellular sucrase activity was detected. Osmotic shock experiments and *TnphoA* mutagenesis indicated that the sucrase enzyme was translocated across the *E. coli* cytoplasmic membrane by a mechanism not involving an N-terminal signal sequence (Scholle *et al.*, 1987; 1989). The presence of sucrase in the supernatant was attributed to non-specific leakage across the outer membrane. Sucrase synthesis in *E. coli* (pVS100) and *V. alginolyticus* was induced by sucrose and repressed by glucose, and the repression was reversed by the addition of cAMP (Scholle *et al.*, 1987). The *V. alginolyticus* sucrose utilization system on plasmid pVS100 was not expressed in an *E. coli crp* mutant (deficient in CAP) and an *E. coli crp cya* mutant (deficient in CAP and adenylate cyclase) (Scholle, 1989). This indicated that at least a functional CAP was required for induction of the sucrose utilization system.

The nt sequence of the *V. alginolyticus* sucrase-encoding gene (*scrB*) has been determined (Scholle *et al.*, 1989). The *scrB* gene consists of a presumptive ATG start codon and a TAA stop codon, and contains 1452 nt encoding 484 aa residues. The  $M_r$  of the predicted polypeptide is 55 657. The deduced aa sequence of the *V. alginolyticus* sucrase enzyme showed 34% and 21% identical aa similarity to the sucrase enzymes from *B. subtilis* and *S. mutans*, respectively.

In contrast to the marine *V. alginolyticus* strain, the collagenolytic *V. alginolyticus* strain and *E. coli* (pVS100) were shown to actively transport sucrose by an  $\text{Na}^+$ -independent sucrose transport system (Scholle *et al.*, 1987). Sucrose was translocated both in the presence and absence of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Li}^+$ . Sucrose uptake was found to be inhibited by the uncoupler CCCP. Synthesis of the sucrose uptake

system was induced by sucrose as was found for the sucrase. The maximum velocity and apparent  $K_m$  values of sucrose uptake for the *V. alginolyticus* strain and *E. coli* (pVS100) were 130 nmol/mg of protein per min and 50  $\mu$ M and 6 nmol/mg of protein per min and 275  $\mu$ M, respectively. It was concluded that in *V. alginolyticus* and *E. coli* (pVS100) cells, sucrose was actively transported, following which it would be hydrolyzed to glucose and fructose by the intracellular sucrase. In *E. coli* (pVS100), the extracellular sucrase would also degrade sucrose to glucose and fructose, which would be taken up by their respective transport systems.

This project represents a continuation of the research on the sucrose utilization system of this collagenolytic *V. alginolyticus* strain. While the results of Scholle *et al.* (1987; 1989) provided extensive insight into the sucrose utilization system of this collagenolytic *V. alginolyticus* strain, a number of areas were highlighted for further investigation. While this collagenolytic *V. alginolyticus* strain and *E. coli* (pVS100) were shown to transport sucrose by a fundamentally different mechanism to that used by a marine *V. alginolyticus* strain, the mechanism of sucrose transport remained unclear and the involvement of the PTS had not been investigated. Thus a molecular analysis of the sucrose transport system of this collagenolytic *V. alginolyticus* strain was needed for a better understanding of the mechanism involved. In addition, such a molecular analysis would allow a closer comparison of this sucrose transport system to those of *B. subtilis*, *S. mutans*, *S. typhimurium* and *K. pneumoniae*, which have been extensively studied at the molecular level and shown to involve the PTS. The sucrose utilization system of *V. alginolyticus* and *E. coli* (pVS100) was shown to be induced by sucrose, and to be subject to catabolite repression which was relieved by the addition of cAMP. The *V. alginolyticus* sucrose utilization system was not expressed in *E. coli* *crp* and *crp cya* mutants. These results together implied the involvement of CAP in the expression of the sucrose utilization system. In addition, the involvement of a pVS100-encoded regulatory protein could not be excluded. A molecular analysis of the regulation of the *V. alginolyticus* sucrose utilization system was needed to investigate these possibilities. This would allow a comparison with the regulatory mechanisms found in the other bacterial sucrose utilization systems, most of which appear to involve regulatory proteins. This collagenolytic *V. alginolyticus* strain has been found to be

similar to *B. subtilis* in a number of physiological aspects (section 1.1.2). In the light of these similarities, the expression of the *V. alginolyticus* sucrose utilization system in *B. subtilis* was an interesting possibility for investigation.

The results of the nucleotide sequencing and molecular analysis of the DNA regions containing the *V. alginolyticus* genes encoding the sucrose uptake system (Blatch *et al.*, 1990) and the regulatory functions are presented. The results of an investigation of expression of the *V. alginolyticus* sucrose utilization system in *B. subtilis* are also presented.

## CHAPTER TWO

### NUCLEOTIDE SEQUENCE AND ANALYSIS OF THE *V. ALGINOLYTICUS* SUCROSE UPTAKE-ENCODING REGION

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## CHAPTER TWO

### NUCLEOTIDE SEQUENCE AND ANALYSIS OF THE *V. ALGINOLYTICUS* SUCROSE UPTAKE-ENCODING REGION

**SUMMARY:** The nt sequence of the DNA fragment containing the *V. alginolyticus* sucrose uptake-encoding region was determined. This region contains two genes, *scrA* and *scrK*. The *scrA* gene encodes a sucrose uptake protein or EII<sup>Scr</sup> protein of the PEP-dependent PTS. The *V. alginolyticus* sucrose utilization system was not expressed in *ptsH* and *crr* strains of *E. coli* indicating that sucrose transport required a functional PTS and was EII<sup>Glc</sup>-dependent. The deduced aa sequence for the *V. alginolyticus* EII<sup>Scr</sup> protein was homologous with the EII<sup>Scr</sup> proteins from *S. mutans*, *S. typhimurium* (pUR400 system) and *B. subtilis*. Transposon *phoA* mutagenesis experiments indicated that the EII<sup>Scr</sup> protein was a membrane-bound protein with a region that extended into the periplasm. The *scrK* gene encoded a fructokinase and the deduced aa sequence was homologous with the *E. coli* enzymes, 6-phosphofructokinase (isoenzyme 2) and ribokinase.

## 2.1 INTRODUCTION

Scholle *et al.* (1987) reported that in a collagenolytic *V. alginolyticus* strain and in *E. coli* cells containing the cloned *V. alginolyticus* sucrose utilization system (pVS100), sucrose was actively transported by a Na<sup>+</sup>-independent uptake mechanism. This sucrose uptake system differed from that reported by Kakinuma and Unemoto (1985) for a marine *V. alginolyticus* strain where sucrose was shown to be taken up by a Na<sup>+</sup>-dependent system. Sucrose transport by this marine *V. alginolyticus* strain did not involve the PTS and sucrose was transported into the cells unchanged. The possible involvement of the PTS in sucrose transport by the collagenolytic *V. alginolyticus* strain has not been investigated.

Bacterial sucrose transport systems were reviewed in the General Introduction (section 1.4). Extensive research has focussed on the sucrose transport systems of the Gram-positive bacteria *B. subtilis* (Fouet *et al.*, 1987) and *S. mutans* (Sato *et al.*, 1989) and the Gram-negative bacteria *S. typhimurium* (pUR400 system) (Ebner and Lengeler, 1988) and *K. pneumoniae* (Sprenger and Lengeler, 1988; Lengeler *et al.*, 1990). In all of these systems sucrose was shown to be transported by an EII<sup>Scr</sup> protein of the PEP-dependent PTS and the *scrA* genes (called *sacP* in *B. subtilis*) encoding the EII<sup>Scr</sup> proteins have been sequenced. Analysis of the deduced aa sequences of these EII<sup>Scr</sup> proteins has revealed the presence of a number of highly conserved sequences and distinct domains (Lengeler *et al.*, 1990). The determination of the nt and deduced aa sequences of the *V. alginolyticus scrA* gene and its product, respectively, would therefore allow a direct comparison with these systems at the level of sequence homology. Such a comparison would give an indication of whether or not sucrose transport in this collagenolytic *V. alginolyticus* strain involves an EII<sup>Scr</sup> of the PTS. The sucrose PTS requires the general PTS components, EI and HPr, before sucrose transport and concomitant phosphorylation can occur. In addition, it was shown that EIII<sup>Glc</sup> played a direct role in the uptake and concomitant phosphorylation of sucrose in *S. typhimurium* (pUR400 system) and *K. pneumoniae* (Lengeler *et al.*, 1982; Sprenger and Lengeler, 1988). An investigation of the expression of the cloned *V. alginolyticus* sucrose utilization system (pVS100) in

*E. coli* strains defective in PTS components would therefore also provide an insight into the possible involvement of the PTS in sucrose transport.

From the aa sequence of the 4 bacterial EII<sup>Scr</sup> proteins a hypothetical prediction of the orientation of these proteins in the cytoplasmic membrane has been proposed (Lengeler *et al.*, 1990). However, direct evidence concerning their orientation in the membrane is not available. The Tn*phoA* system developed by Manoil and Beckwith (1985) has been used successfully to investigate the membrane topology of membrane-bound proteins (Chun and Parkinson, 1988; Lloyd and Kadner, 1990). The alkaline phosphatase moiety encoded by Tn*phoA* lacks its own signal peptide rendering it inactive since it can no longer be exported to the periplasm. Alkaline phosphatase may be inactive unless exported to the periplasm because of its inability to form dimers, acquire zinc ions or form proper intrachain disulphide bonds. It has been found that PhoA fusions to different regions of a membrane-bound protein displayed different levels of alkaline phosphatase activity. Fusions with high activity are considered to be those in which the PhoA segment is linked to a part of the membrane-bound protein exposed to the periplasm, whereas low-activity hybrid proteins result from fusions to cytoplasmic domains of the membrane-bound protein. The Tn*phoA* system is therefore a suitable tool for a preliminary investigation of the membrane topology of the *V. alginolyticus* sucrose transport protein.

The DNA sequence determination and molecular analysis of the cloned *V. alginolyticus* sucrose uptake-encoding region adjacent to the sucrase-encoding gene (*scrB*) will be discussed in this chapter.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Bacterial strains, plasmids and phages

The source of the genetic material used in this study was a collagenolytic *V. alginolyticus* strain (NCIB 11038) originally isolated from hides (Welton and Woods, 1973; 1975) (section 1.1.2).

*E. coli* strains are described in Table 2.1. *E. coli* JA221 transformants were used for all sucrose, fructokinase and sucrose uptake assays. *E. coli* LK111 served as a recipient for exonuclease III shortened plasmids. Studies on the PTS-dependence of sucrose transport were done with *E. coli* 1100, 1101, 3.300, DF51 and *ctr-7*. Studies on the EIII<sup>Glc</sup>-dependence of sucrose transport were done with *E. coli* JWL314, LR2-67 and JLV86 (gifts from J.W. Lengeler, Fachbereich Biologie/Chemie, Universitat Osnabruck, FRG). The PhoA<sup>-</sup> *E. coli* strain CC118 was used for the Tn*phoA* fusion experiment. Phage  $\lambda$ b221*rex::TnphoA* cI857 Pam3 was a gift from C. Manoil (Gutierrez *et al.*, 1987) (Appendix B).

Plasmids pVS100, pVS104 and pVS110 have been described previously and will be given a brief introduction here. A genomic library of *V. alginolyticus* DNA was prepared by cloning *sau3A* digested chromosomal DNA into *Bgl*II restriction endonuclease digested pEcoR251 (Maharaj *et al.*, 1986). Plasmid pVS100 originates from this genomic library and contains the *V. alginolyticus* sucrose utilization system cloned on a 10.4-kb chromosomal fragment (Scholle *et al.*, 1987). The 6.5-kb *Pst*I(vector)-*Hind*III restriction endonuclease fragment of pVS100 was subcloned into *Pst*I-*Hind*III restriction endonuclease digested Bluescript SK (Stratagene, San Diego) to generate pVS104 (Scholle *et al.*, 1989) (Figs 1.9 [section 1.4.4] and 2.1). Plasmid pVS110 was constructed by deleting the 2.7-kb *Hpa*I-*Hind*III restriction endonuclease fragment from pVS104 (Scholle *et al.*, 1989) (Figs 1.9 [section 1.4.4] and 2.1). The M13-derived Bluescript SK plasmid (Stratagene, San Diego) was used for the preparation of nt sequencing templates (Appendix B). Plasmids pVS103, pVS114 and pVS115 were constructed in this study.

TABLE 2.1 *E. coli* strains used in this study

Strain	Genotype	Reference
JA221	F <sup>-</sup> <i>recA1 leuB6 trpE5</i> <i>hsdR lacY λ<sup>-</sup></i>	Beggs (1978)
LK111	a <i>lacI<sup>q</sup> lacZΔ M15 lacY<sup>+</sup></i> derivative of K514	Zabeau and Stanley (1982)
1100	<i>thi-1 relA1 bglR11 λ<sup>-</sup></i>	Fox and Wilson (1968)
1101	<i>his-62 ptsH1 relA1 bglR11 thi-1</i> <i>λ<sup>-</sup> spoT1</i>	Fox and Wilson (1968)
3.300	<i>lacI22 λ<sup>-</sup> relA1 spoT1 thi-1</i>	Pardee <i>et al.</i> (1959)
DF51	<i>lacI22 dctB3 λ<sup>-</sup> ptsI2 relA1 spoT1</i> <i>thi-1</i>	Lo <i>et al.</i> (1972)
<i>ctr-7</i>	<i>ptsI7 relA1 bglR10 thi-1 λ<sup>-</sup> spoT1</i>	Morse <i>et al.</i> (1971)
JWL314	F <sup>-</sup> <i>argG6 metB1 hisG1 rpsL104</i> <i>tonA2 supE44 DphoA8 galT6</i> <i>xyl-7 gatR49 malT1 k<sup>R</sup></i>	Lengeler (1980) Lengeler <i>et al.</i> (1981)
LR2-167	as JWL314 and <i>manI161</i> <i>manA162 nagE167 mal<sup>+</sup> k<sup>S</sup></i>	Lengeler (1980) Lengeler <i>et al.</i> (1981)
JLV86	as LR2-167 and <i>crr-1 zfb::Tn10 Tc<sup>R</sup></i> Succ <sup>-</sup>	Vogler <i>et al.</i> (1988)
CC118	<i>araD139 Δ(ara,leu)7697 ΔlacX74</i> <i>phoAΔ20 galE galK thi rpsE rpoB</i> <i>argE<sub>am</sub> recA1</i>	Manoil and Beckwith (1985)

## 2.2.2 Standard methods, media, buffers and solutions

Standard methods, media, buffers and solutions are described in Appendix A.

### 2.2.3 Subcloning the sucrose utilization system

The 4.4-kb *HindIII*(vector)-*HindIII* restriction endonuclease fragment of pVS100 was deleted and the resulting 9.5-kb *HindIII*-*HindIII* restriction endonuclease fragment was self-ligated to generate pVS103 which still contained the entire sucrose utilization system (same insert fragment as pVS104; Fig. 2.1). The 2.6-kb *BglIII*-*ClaI* restriction endonuclease fragment of pVS104 was gel purified (Appendix A) and subcloned into *Bam*HI-*ClaI* restriction endonuclease digested Bluescript to generate pVS114 (Fig. 2.1). The 1.7-kb *EcoRV*-*EcoRV* restriction endonuclease fragment of pVS104 was gel purified (Appendix A) and subcloned into *EcoRV* restriction endonuclease digested Bluescript SK to give pVS115 (Fig. 2.1). In all subcloning experiments, the DNA used was purified by isopycnic CsCl-EtBr density gradient ultracentrifugation (Appendix A). Restriction endonuclease digestions were carried out in a final volume of 20  $\mu$ l as described in Appendix A. Apart from the gel purified DNA fragments, the restriction endonuclease digestion products were purified by one phenol extraction and several ether extractions followed by an ethanol precipitation in the presence of 0.3 M sodium acetate before being ligated and transformed into *E. coli* (Appendix A). Transformants were selected on LB or sucrose MacConkey agar containing ampicillin (Ap) (100  $\mu$ g/ml) (Appendix A). Plasmid DNA was isolated from transformants by the small scale procedure and the presence of the desired restriction endonuclease fragments verified by restriction endonuclease digestion and 0.8% agarose gel electrophoresis (Appendix A). Plasmids to be used extensively were isolated by the large scale method and purified by isopycnic CsCl-EtBr density gradient ultracentrifugation (Appendix A).

### 2.2.4 Exonuclease III digestion

Exonuclease III deletions of pVS114 were generated by a modification of the method of Henikoff (1984). Progressive deletions from the 5' end of the insert were generated by unidirectionally digesting *KpnI*-*AccI* restriction endonuclease digested pVS114 with exonuclease III. Similarly, deletions were made from the 3' end of the insert using *Bst*XI-*XbaI* restriction endonuclease digested pVS114. DNA (10  $\mu$ g), purified by isopycnic CsCl-EtBr density gradient ultracentrifugation, was digested

with 20 units of each of the respective restriction endonucleases (Appendix A). The digestion products were purified, ethanol precipitated (section 2.2.3) and the DNA pellet resuspended in 100  $\mu$ l 1 x exobuffer. Exonuclease III digestion was carried out as described in Appendix A. The deletions were transformed into *E. coli* LK111 and transformants were selected on LB agar containing Ap (100  $\mu$ g/ml) (Appendix A). Shortened pVS114 plasmids were isolated by the small scale procedure and sized by 0.8% agarose gel electrophoresis (Appendix A). Useful DNA sequencing templates were selected, isolated by the large scale method and purified by isopycnic CsCl-EtBr density gradient ultracentrifugation (Appendix A).

### 2.2.5 Nucleotide sequencing

The nt sequence of both strands of the DNA upstream of the *scrB* gene was determined using the overlapping DNA fragments generated by exonuclease III digestion of pVS114. DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method described in Appendix A.

The DNA and deduced aa sequences were analyzed on an IBM XT computer using the Genepro programme version 4.1 (Riverside Scientific) and the Genetics Computer Group Incorporated Sequence Analysis Software Package version 6.1 (GCG Package). All the current databases accompanying the GCG Package were screened for related nt and aa sequences.

### 2.2.6 $\lambda$ ::*TnphoA* mutagenesis

Transposon insertions into pVS103 were obtained by a modification of the method of de Bruijn and Lupski (1984). Phage  $\lambda$ ::*TnphoA* at a multiplicity of infection of approximately 1 was added to late stationary phase *E. coli* CC118 (pVS103) cells in LB (2 ml) containing 10 mM MgSO<sub>4</sub> and incubated at 30°C for 15 min. The culture was subsequently diluted 10 fold with LB and incubated for a further 3 h at 30°C with good aeration. The cells were concentrated and transposon insertions selected on LB agar, supplemented with sucrose (0.2%, w/v), Ap (100  $\mu$ g/ml), kanamycin (Km) (250  $\mu$ g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (XP) (40  $\mu$ g/ml). After

incubation at 30°C for 2 to 3 days, blue colonies (alkaline phosphatase-positive) were selected for the preparation of plasmid DNA by the small scale method (Appendix A). This DNA was used to transform *E. coli* CC118 and the plasmids able to transform *E. coli* CC118 to blue, alkaline phosphatase-positive colonies were chosen for large scale plasmid preparation, mapping and sequencing (Appendix A). A 15-bp synthetic oligonucleotide primer, 5'-AAACGGCGAGCACCG-3', was used to provide nt sequence across the junction of the *phoA* gene and pVS103 (Appendix A). The primer was complementary to the nt sequence corresponding to nt positions 126-140 of the *phoA* gene.

### 2.2.7 Enzyme assays

Enzyme assays were carried out on late-exponential-phase cultures of *E. coli* and *E. coli* transformants ( $A_{600}=1.0$ ). Samples were assayed in duplicate and experiments were repeated three times.

Sucrase and fructokinase assays were carried out on cell extracts. Cells (20 ml) grown at 37°C in minimal medium (MM; Appendix A) supplemented with an appropriate carbon source and Ap (50 µg/ml) were harvested by centrifugation at 12000 × g for 7 min at 4°C. The pellet was washed twice with saline solution (0.87% NaCl, w/v) before being resuspended in 0.1 M Tris-HCl buffer (2 ml) (pH 7.0 sucrase assays; pH 7.5 fructokinase assays). The cell suspension was sonicated in a MSE Soniprep 150 for three 30 s bursts at 4°C with a 30 s interval after each burst. The cell debris was removed by centrifugation in an Eppendorf microfuge at 4°C for 15 min and the supernatant fluid was retained for enzyme assays.

Sucrase and fructokinase activities were determined for *E. coli* JA221 and *E. coli* JA221(pVS100) cells grown in MM supplemented with either sucrose (plus 1% glycerol, v/v, for *E. coli* JA221), glucose or fructose (Appendix A). In addition fructokinase activity was determined for *E. coli* JA221(SK) and *E. coli* JA221(pVS115) cells grown in MM supplemented with glucose (Appendix A).

**SUCRASE:** Sucrase activity was assayed by a modification of the method described by Scholle *et al.* (1987). Sucrase activity was determined by incubating 15  $\mu$ l of 0.88 M sucrose in 0.1 M Tris-HCl buffer (pH 7.0) with 35  $\mu$ l of an appropriate enzyme dilution at 37°C for 30 min. The enzyme reaction was terminated by adding 150  $\mu$ l dinitrosalicylic acid reagent (DNS) (Miller, 1959) (Appendix A) and boiling the mixture for 5 min. Control reactions, which were terminated at  $t=0$  by the addition of DNS, were prepared for each sample. The amount of reducing sugar released was determined spectrophotometrically at 510 nm. The absorbance readings at 510 nm ( $A_{510}$ ) were converted to  $\mu$ moles reducing sugar per ml using a standard curve prepared by plotting  $A_{510}$  against known concentrations of glucose solutions. Sucrase specific activity was expressed as  $\mu$ moles of reducing sugar produced per min per mg protein.

**FRUCTOKINASE:** Fructokinase activity was assayed by the direct method of Sprenger and Lengeler (1988). The reaction mixture (100  $\mu$ l) contained in 0.1 M Tris-HCl buffer (pH 7.5): 5 mM adenosine 5'-triphosphate (ATP), 10 mM  $MgCl_2$ , 35  $\mu$ M [ $^{14}C$ ]fructose (290 mCi/mmol) and an appropriate enzyme dilution. Incubation was at 30°C and samples (10  $\mu$ l) were removed at regular intervals, mixed with 20% fructose (2  $\mu$ l), placed onto an ion exchange filter (Whatman DE81) and air dried. Air dried filters were washed with sterile distilled water (2 ml), air dried and counted in toluene-based scintillant (5 ml) in a Beckman LS1701 Liquid Scintillation System. To determine the total counts present in each reaction mixture, an unwashed sample (10  $\mu$ l) was counted in the presence of scintillant (5 ml). Fructokinase specific activity was expressed as nmoles of phosphorylated fructose produced per min per mg protein.

**ALKALINE PHOSPHATASE:** Alkaline phosphatase activity was assayed by an adaptation of the method of Brickman and Beckwith (1975). *E. coli* CC118 transformants (20 ml) were grown at 37°C in LB supplemented with sucrose and Ap (100  $\mu$ g/ml; *E. coli* CC118(pVS103)) or Ap and Km (250  $\mu$ g/ml; *E. coli* CC118(pVS103::Tn $phoA$ )). Cell samples (1 ml) to be assayed were centrifuged in an Eppendorf microfuge for 30 s at 4°C and the pellet washed twice with saline solution (0.87% NaCl, w/v). An appropriate dilution of the cell sample was made in

1 M Tris-HCl buffer (pH 8.0). The diluted cell sample (1 ml) was mixed with chloroform (50  $\mu$ l) and 0.1% SDS (50  $\mu$ l) and equilibrated for 5 min at 37°C. The alkaline phosphatase substrate (4 mg/ml *p*-nitrophenyl phosphate (PNPP) in 1 M Tris-HCl buffer pH 8.0) (100  $\mu$ l) was added and the time taken for the sample to turn light yellow was accurately recorded. The reaction was terminated by the addition of 1 M  $\text{KH}_2\text{PO}_4$  (100  $\mu$ l) and the samples were spun in an Eppendorf microfuge for 1 min at 4°C. The supernatant was removed and its absorbance at 420 nm ( $A_{420}$ ) recorded. The cell samples prepared from *E. coli* CC118(pVS103) were used as control samples. Alkaline phosphatase specific activity was expressed as units/ $A_{600}$  using the formula:

$$\text{units}/A_{600} = 1000 \times \frac{A_{420}}{A_{600} \times \text{min}} \times \text{dilution factor.}$$

**SUCROSE UPTAKE:** Uptake of [ $^{14}\text{C}$ ]Sucrose was assayed as described by Scholle *et al.* (1987). Sucrose uptake was determined for *E. coli* JA221(pVS100) cells grown at 37°C in MM supplemented with sucrose or glucose and Ap (50  $\mu\text{g}/\text{ml}$ ) (Appendix A). Cells (20 ml) were harvested by centrifugation at 6000  $\times g$ , washed twice in 1  $\times$  CSH MM salts and resuspended in an equal volume of the same solution (Appendix A). All cells were kept at room temperature throughout the experiment and samples of the cell suspension were retained for protein concentration determinations. Uptake of sucrose was determined in the presence of 15 mM glucose and 15 mM fructose after an adaptation period of 30 s. Uptake of [ $^{14}\text{C}$ ]sucrose (specific activity 10 Ci/mol, Amersham) was started by the addition of 250  $\mu\text{M}$  (final concentration) of labelled sucrose to 400  $\mu\text{l}$  of cell suspension. Samples (50  $\mu\text{l}$ ) were withdrawn and filtered through membrane filters (type HA, pore size 0.45  $\mu\text{m}$ ; Millipore Corp.) at regular time intervals over 21 min. Membrane filters were presoaked in sterile, distilled water for 14 h before use, and rinsed with CSH MM salts (2 ml) after filtration. After being dried, the filters were counted in toluene-based scintillant (5 ml) in a Beckman LS1701 Liquid Scintillation System. To determine the total counts present in each uptake reaction mixture, an unfiltered sample (50  $\mu\text{l}$ ) was counted in the presence of scintillant (5 ml). [ $^{14}\text{C}$ ]sucrose uptake was expressed as nmoles [ $^{14}\text{C}$ ]sucrose per min per mg of protein.

### 2.2.8 Protein assays

Protein concentrations were determined by the method of Bradford (1976). Assays were performed in triplicate using disposable cuvettes. Bradford's reagent (1 ml; Appendix A) was added to 100  $\mu$ l of sample and mixed well. The mixture was kept at room temperature for 5 min before reading the absorbance at 595 nm. Standard curves ranging from 0-200  $\mu$ g/ml were prepared using bovine serum albumin.

### 2.2.9 Studies on the PTS- and EIII-dependence of sucrose transport

Plasmid pVS100 was transformed into the *ptsI*, *ptsH* and *crr* strains of *E. coli* and their corresponding parent strains (Table 2.1). The ability of the transformants to transport and metabolize sucrose was determined by their growth and colour reaction on sucrose MacConkey agar plates containing Ap (100  $\mu$ g/ml) and their growth on sucrose MM agar plates containing Ap (100  $\mu$ g/ml) (Appendix A). Growth and colour reactions were scored after 24 h growth at 37°C. The effect of adding cAMP (1 mM final concentration) to the growth medium was also investigated.

For the *crr* strain of *E. coli* and its parent strains, the growth of the transformed cells was also investigated on various PTS, class I and class II sugars (categories defined for enteric bacteria in section 1.3.2 of the General Introduction). Growth and colour reaction was scored after 24 h growth at 37°C on MacConkey agar plates containing Ap (100  $\mu$ g/ml) and: (i) PTS sugars, sucrose or glucose; (ii) class I sugars, glycerol or lactose and (iii) class II sugar, rhamnose. In each case the effect of adding cAMP (1 mM final concentration) to the growth medium was also investigated.

### 2.2.10 DNA-directed cell-free protein synthesis

The synthesis of proteins by plasmids pVS103, pVS110, pVS114, and pVS115 was investigated in an *E. coli* DNA-directed cell-free system (Amersham, Prokaryotic DNA-directed cell-free translation kit, Code N.380). The manufacturer's protocol was followed throughout using quarter quantities. The polypeptides were labelled

with L-[<sup>35</sup>S]methionine (specific activity 1040 Ci/mmol) and resolved on 12% SDS-PAGE gels (Laemmli, 1970; O'Farrell, 1975) (Appendix A). The following low molecular weight markers (Pharmacia;  $M_r$  in parenthesis) were used: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and  $\alpha$ -lactalbumin (14 400). The *in vitro* translated polypeptides were visualized by autoradiography.

## 2.3 RESULTS AND DISCUSSION

Scholle *et al.* (1989) showed that the *V. alginolyticus* sucrose uptake-encoding gene (*scrA*) and the sucrose-encoding gene (*scrB*) were present on plasmid pVS104, with the *scrA* gene located at the 5' region and the *scrB* gene at the 3' region of pVS104 (section 1.4.4 of the General Introduction). The *scrB* gene has been sequenced (Scholle *et al.*, 1989) and the extent of sequencing reported is indicated in Fig. 2.1.

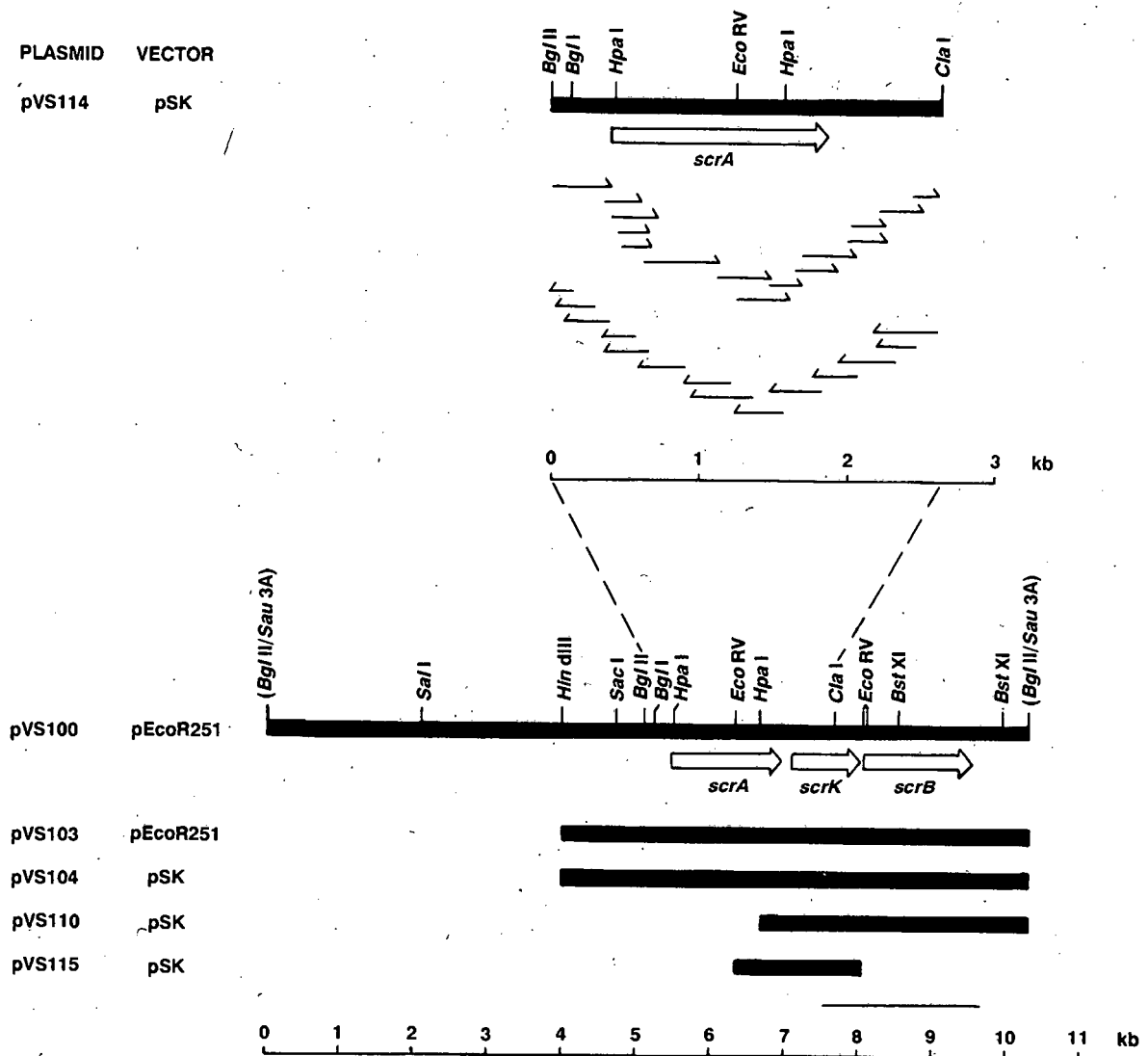


FIG. 2.1 Restriction map of pVS100 insert DNA, subclones derived from pVS100 and the nucleotide sequencing strategy for the *V. alginolyticus* sucrose uptake-encoding region contained on pVS114. The bold lines, single thin line and open arrows represent *V. alginolyticus* insert DNA, the extent of sequencing previously reported (Scholle *et al.*, 1989) and the ORF's, respectively. The thin arrows represent the extent and direction of sequencing of templates generated by exonuclease III digestion.

DNA sequencing templates generated by exonuclease III digestion from the 5' and 3' ends of the insert in plasmid pVS114 were constructed and overlapping sequence data were obtained for both DNA strands of the insert (Fig. 2.1). The region of overlap with the upstream region of the *scrB* gene was identified and the resulting sequence found to contain a sucrose uptake-encoding gene (*scrA*) and a fructokinase-encoding gene (*scrK*) (Figs 2.1 and 2.2).

### 2.3.1 Molecular analysis of the *V. alginolyticus scrA* gene

**NUCLEOTIDE SEQUENCE:** The *scrA* gene consists of a presumptive ATG start codon and a TAA stop codon, and contains 1437 bp which encodes a polypeptide of 479 aa residues (Fig. 2.2). The  $M_r$  of the predicted polypeptide is 49 889. The ATG start codon is not preceded by a classical ribosome-binding site (Shine and Dalgarno, 1974). A GGGG sequence is located 6 bp upstream of the ATG start codon (Fig. 2.2). A promoter-like sequence (Hawley and McClure, 1983) is located upstream of the ATG start codon (TTGATA-N<sub>19</sub>-TATACT; Fig. 2.2). An inverted repeat structure which might act as a Rho-independent transcriptional termination loop (Rosenberg and Court, 1979) is located 50 bp downstream of the TAA stop codon. The free energy of this potential stem loop structure was calculated to be -24.6 kcal/mol (Salser, 1977).

**DEDUCED AA SEQUENCE AND HOMOLOGY COMPARISONS:** Analysis of the deduced aa sequence of the *scrA* gene product indicated that the *scrA* gene encoded a sucrose uptake protein or EII<sup>Scr</sup> protein of the PEP-dependent PTS. On the basis of identical aa similarities (Fig. 2.3) the *V. alginolyticus* EII<sup>Scr</sup> protein showed 46, 42, and 38% similarity to the *S. mutans* EII<sup>Scr</sup> (first 488 aa), the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> and the *B. subtilis* EII<sup>Scr</sup> proteins, respectively.



Va	M N Y P A V A K E L L T L L G G R S N I T A L A H C A T R L R L L A V A D E Q K I D E Q A I D N L E G V R G Q F K V A G Q	60
Sm 46%	M D Y S K V A S E V I T A V G R D N L V A A A H C A T R L R L L V L R D D S K V D Q R A L D K N A D V K G T F K T D G Q	59
St 42%	M D F E Q I S C S L L P L L G G R E N I - A S A H C A T R L R L L V L V D D S L A D Q Q A I G K V E G V K G C F R N A G Q	59
Bs 38%	M D Y K E T A K R L I E L L G G R E N I S A A H C A T R L R L L V N K D E S K I D Q A Q V E E L D G V R G A F S S S G Q	60
▼		
Va	Y Q I I F G S G I V N Q V Y A E M A R K L T G M S E M S T N D V A S A G A E - - K Q N I V Q P A V K G L S D I F V P I I P	118
Sm	Y Q V I I G P G D V N F V Y D E I I R Q T G L T E V S T D D L K K I A A S G R K K F N P I M A L I K L L S D I F V P I I P	119
St	M Q I I F G T G V V N K V Y A A F T Q A A G I S E S S K S E A A D I A A - - K K L N P F Q R I A R L L S N I F V P I I P	117
Bs	Y Q I I F G T G L V N K V F D A F S K E A D I E R E E H V - - N H Q D A A K E K L N P A A R F A K T L S N I F V P I I P	118
▼		
Va	A I V A G G L L M G I Y N L L T A Q G L F I D G K S L I E A N P G L T D L A N M I N T F A N A P F V Y L P I L L A F S A	178
Sm	A L V A G G L L M A L N N F L T S E G L F G T - K S L V Q O F P I I K G S S D M I Q L M S A A P F W F L P I L V G I S A	178
St	A I V A S G L L M G L L G M V K T Y G W V D P G N A I Y I - - - - - M L D N C S S A A F I I L P I L I G F T A	167
Bs	A I V A S G L L M G L L G M I N A F H W M S K D S A L L Q L - - - - - L D M F S S A A F I F L P I L I G V S A	168
▼		
Va	S K K F G G N P Y L G A A L G M L K V H P D L L N G W G F G G A S V S G N I P - - - - - V W N I L G F E I Q K V G Y	231
Sm	A K R F G A N Q F L G A S I G M I H V A P G A A N I I G L A A N A P I S K A A T I G A Y T G F W N I P G L H V T Q A S Y	238
St	A K E F G G N P Y L G A T L G G I L T H P A L T N A W G V A A G F H - - - - - T M N P F G F E I A M I G Y	215
Bs	S K E F G S N P Y L G A V I G G I M I H P N L L N P W G L A E E Q L I - - - - - T C I F S D L I S L P S A	216
▼		
Va	Q G S V L P V L V S A P I L A K V E L G R K K V I P S V L D N L L T P L L A I P I A G L L T F T V V G P F T R D I G F L	291
Sm	T Y Q V I P V L V A V W L L S I L E K P F H K R L P S A V D F T P T P L L S V I I T G F L T F I V I G P V N K E V S D W	298
St	Q G T V P P V L L A V W F M S I V E K Q L R R A I P D A L D L I L T P P L T V I I S G F I A L L I I G P A G R A L G D G	275
Bs	T G N C Y P C P A C G V C D E Q G R E N D E K S G S T C G D L L V T P F V T V I V T G F V A P I A I G P L G R A L G S G	276
▼		
Va	L G D G L N W L Y N T A G F V G G A V P G L I Y A P F V I T G M H R S P I A I E T Q L L A D I A T - T G - G T P I F P I	349
Sm	L T N G I V W L Y D T T G F L G M G V F G A L Y S P V V M T G L M Q S P P A I E T Q L I S A P Q N G T G H G D P I F V T	358
St	I S P V L S T L I S H A G W L A G L L F G G L Y S V I V I T G I M H S P F R A V E A G L L G N P S I G V - - N P L L P I	332
Bs	I T V A L T Y V Y D H A G P V A G L I P G G T Y S L I V L T G V M H S P F R A I E A G L I A D I G K N Y L L - - - - P I	331
▼		
Va	A A H S N V S Q G A A A L A V G V M S K D K K K G I A I P S G V T G L L G I T E P A H P G V N L K L R Y P P I A A V C	409
Sm	A S M A N V A Q G A A T P A I Y F L T K D K K K G L S S S G V S A L L G I T E P A L P G V N L K Y R P P F F C A L I	418
St	W A H A N V A Q G G A C L A V W F P T K D A K I X A I T L P S A F S A H L G I T E A A I P G I N L R P V K P P I A A L I	392
Bs	W S M A N V A Q G G A G L A V P P H A K K A R T K E I A L P A A P S A P L G I T E P V I P G V N L R Y R K P P I A A N I	391
▼		
Va	A A A L S S A F I T M P N V K A Q A L G A A G L P G I I S I T P D K I G Y Y I A G N V I A P L T A F V L T I V L G I G D	469
Sm	G S A S A A A I A G L L O V V A V S L G S A G F L G P L S I K A S S I P P Y V V C E L I S P A I A F A V T Y G Y G K T K	478
St	G G A A G G A W V V S V H V Y M T A V G L T A I P G M A I V Q A S S L N Y I I G H V M P L A S P L R C S L V L K Y K T	452
Bs	G G A L G G A Y V V P T H V A A N A Y G L T G I P R I A I A A P F G P S N L I H Y L I G N A I A A V S A P I A A P V H K	451
▼		
Va	R A K V G K K A A A *	479
Sm	A V D V F A A E A A	488
St	D A E *	456
Bs	I N E D E E R K K *	460

FIG. 2.3 Comparison of the *V. alginolyticus* EII<sup>Scr</sup> protein (Va) with the *S. mutans* EII<sup>Scr</sup> protein (Sm) (first 488 aa), the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein (St) and the *B. subtilis* EII<sup>Scr</sup> protein (Bs). The aa are identified by the single-letter code and identical aa are boxed. The highly conserved cysteine residue, histidine residue and GITE motif are in boldface type and indicated by closed arrowheads. The numbers preceding each sequence indicate the percentage identical aa similarity of the *V. alginolyticus* EII<sup>Scr</sup> protein to each EII<sup>Scr</sup> protein. The numbers at the end of each line represent the positions of the aa in each sequence. Asterisks indicate the C-termini of the proteins.

Genetic analysis and comparison of several EII proteins from Gram-positive and Gram-negative bacteria has allowed the identification of 3 domains (Lengeler *et al.*, 1990; section 1.3.1 of the General Introduction): (i) an EIII-like domain, (ii) an EIII-binding domain and (iii) a hydrophobic domain. Of the EII<sup>Scr</sup> proteins studied to date only the *S. mutans* EII<sup>Scr</sup> protein has been found to contain an EIII-like domain suggesting that it was EIII-independent (Sato *et al.*, 1989). Sucrose transport and concomitant phosphorylation by *S. typhimurium* (pUR400 system) and *K. pneumoniae* has been shown to require EIII<sup>Glc</sup>. This indicated that the *S. typhimurium* (pUR400 system) and *K. pneumoniae* EII<sup>Scr</sup> proteins were EIII<sup>Glc</sup>-dependent forming EII<sup>Scr</sup>/EIII<sup>Glc</sup> pairs (Lengeler *et al.*, 1982; Sprenger and Lengeler, 1988). Analysis of the deduced aa sequence of the *S. typhimurium* EII<sup>Scr</sup> protein confirmed this result since no EIII-like domain was found. Analysis of the deduced aa sequence of the *V. alginolyticus* EII<sup>Scr</sup> protein indicated that it did not contain an EIII-like domain suggesting that it was an EIII-dependent EII<sup>Scr</sup> protein most likely interacting with EIII<sup>Glc</sup>. The *V. alginolyticus* EII<sup>Scr</sup> protein was found to contain regions which corresponded to the EIII-binding domain and hydrophobic domain proposed for other EII proteins (Fig. 2.3).

The *V. alginolyticus* EII<sup>Scr</sup> protein was found to contain a region at its N-terminus (aa residues 1-76) which was highly homologous to the N-terminal regions proposed to be the EIII-binding domains of the other EII<sup>Scr</sup> proteins (Fig. 2.3). The critical cysteine residue proposed to be involved in phosphoryl transfer was conserved at position 26 and was situated in a block of high homology (Fig. 2.3). In addition, the N-terminus of the *V. alginolyticus* EII<sup>Scr</sup> protein was found to contain 2 amphipathic, potentially helical leader sequences (aa residues 1-14 and 20-30; Fig. 2.4). N-terminal, amphipathic, potentially helical leader sequences have been proposed for the other 3 EII<sup>Scr</sup> proteins and are thought to target the EII<sup>Scr</sup> proteins to the cytoplasmic membrane (Saier *et al.*, 1989). The first N-terminal helix present in the *V. alginolyticus* EII<sup>Scr</sup> protein resembled that proposed for the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein. In both, the hydrophilic half of the helix was not highly charged and contained a helix breaking proline residue.



The N-terminus of the *V. alginolyticus* EII<sup>Scr</sup> protein was also found to contain 2 relatively large hydrophilic regions (aa residues 25-56 and 76-111; Figs 2.3 and 2.5) separated by a hydrophobic region. The second hydrophilic region was followed by the hydrophobic domain (Figs 2.3 and 2.5).

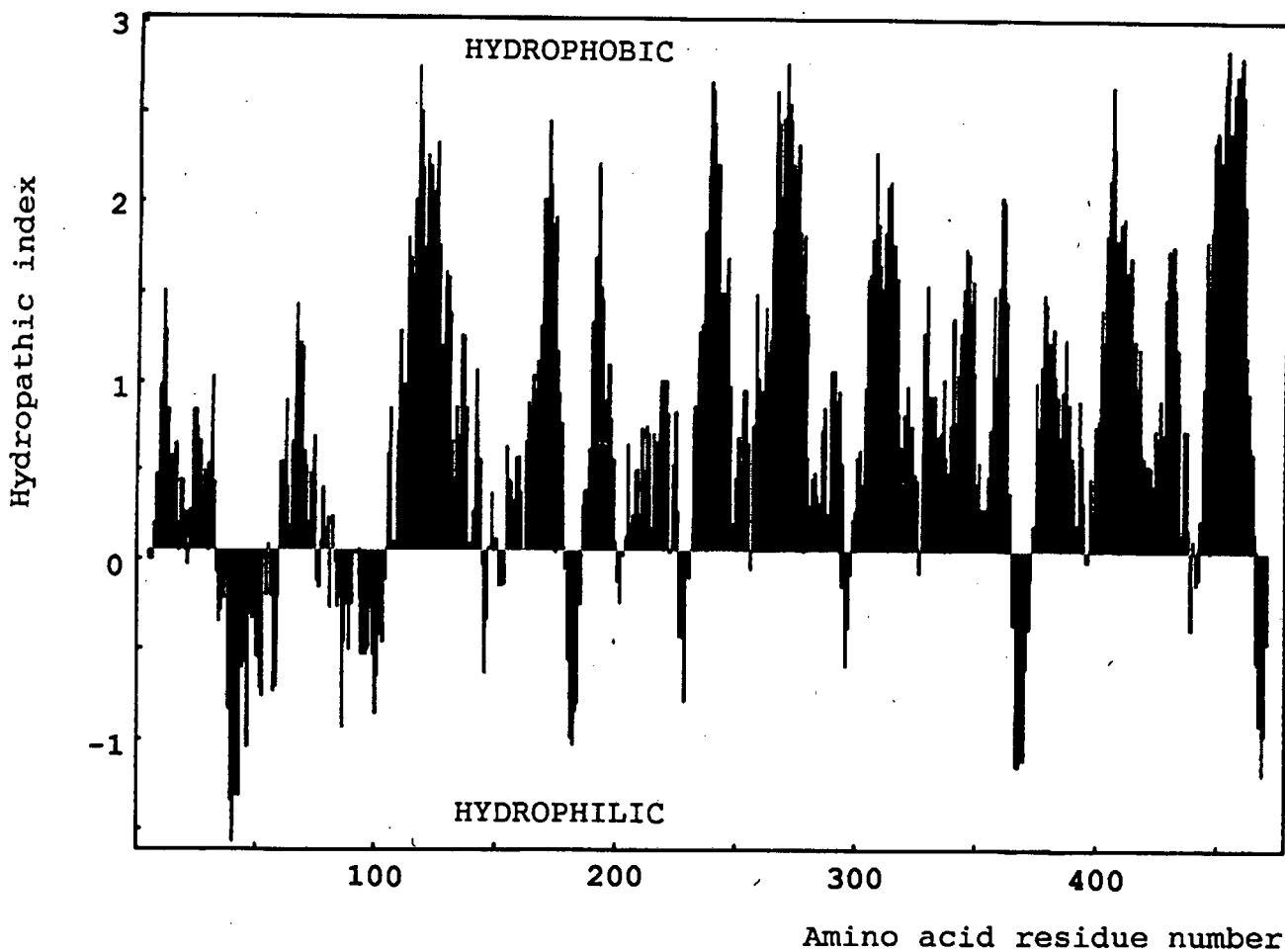


FIG. 2.5 Hydropathy plot of the *V. alginolyticus* EII<sup>Scr</sup> protein (Kyte and Doolittle, 1982). The window size chosen was 10 aa.

The hydrophobic domain was found to contain a number of highly conserved blocks especially the blocks containing the highly conserved histidine residue (His309) and the GITE motif (aa residues 387-390) (Fig. 2.3). His309 and the GITE motif (or a similar motif) have been located at similar positions in a number of EII proteins, however, their functions remain to be elucidated (Bramley and Kornberg, 1987; Saier *et al.*, 1988; Lengeler *et al.*, 1990). An amphipathic helix has been found at the start of the hydrophobic domain in a number of EII proteins (Lengeler *et al.*, 1990),

however, a strong amphipathic helix was not found at the start of the hydrophobic domain of the *V. alginolyticus* EII<sup>Scr</sup> protein. The hydrophobic domain was terminated with a series of charged aa as has been found for other EII proteins (Lengeler *et al.*, 1990).

Those EII proteins that function without an EIII and all EIII proteins are terminated at the C-terminus by a hydrophobic residue followed by two charged residues, one of which is always a lysine or an arginine residue (Saier *et al.*, 1988). This was not the case for the *V. alginolyticus* EII<sup>Scr</sup> protein further suggesting that it was EIII-dependent.

The hydropathy plot (Kyte and Doolittle, 1982) for the deduced aa sequence of the *scrA* gene product indicates that the *V. alginolyticus* EII<sup>Scr</sup> is a relatively hydrophobic protein (Fig. 2.5). This protein contains 56% non-polar, 31% polar, 7% positively charged and 6% negatively charged aa. This result is characteristic of an integral membrane protein and is similar to that reported for the EII<sup>Scr</sup> proteins from *S. typhimurium* (pUR400 system; Ebner and Lengeler, 1988) and from *S. mutans* (Sato *et al.*, 1989).

**PTS- AND EIII-DEPENDENCE OF SUCROSE TRANSPORT:** The results in Table 2.2 indicated that the *V. alginolyticus* sucrose utilization system was not expressed in the *E. coli ptsH* strain (1101) and was lethal in the *ptsI* strains (DF51 and *ctr-7*). It has been found that *ptsH* strains of *E. coli* are unable utilize a number of non-PTS carbohydrates, but that this defect was relieved by the addition of exogenous cAMP (Postma and Lengeler, 1985; section 1.3.2 of the General introduction). The lack of expression of the *V. alginolyticus* sucrose utilization system in the *E. coli ptsH* strain could not be relieved by the addition of cAMP. The respective parent strains expressed the *V. alginolyticus* sucrose utilization system with an identical phenotype to that reported for *E. coli* JA221(pVS100). These results suggested that sucrose transport by *E. coli* strains transformed with the *V. alginolyticus* sucrose utilization system required a functional PTS. These results were consistent with the sequence comparisons that strongly suggested that the *V. alginolyticus scrA* gene encoded an EII<sup>Scr</sup> protein.

**TABLE 2.2** Phenotypes of *ptsH*, *ptsI* and their respective parent strains of *E. coli* transformed with the *V. alginolyticus* sucrose utilization system on plasmid pVS100. Growth was determined on sucrose MM agar plates and sucrose MacConkey agar plates

Strain	Relevant Genotype	Phenotype <sup>a</sup>	
		MM	MacConkey <sup>b</sup>
1100(pVS100)	<i>ptsH</i> <sup>+</sup> <i>ptsI</i> <sup>+</sup>	3+	3+
1101(pVS100)	<i>ptsH</i> <i>ptsI</i> <sup>+</sup>	-	-
3.300(pVS100)	<i>ptsH</i> <sup>+</sup> <i>ptsI</i> <sup>+</sup>	3+	3+
DF51(pVS100)	<i>ptsH</i> <sup>+</sup> <i>ptsI</i>	L	L
<i>ctr-7</i> (pVS100)	<i>ptsH</i> <sup>+</sup> <i>ptsI</i>	L	L

<sup>a</sup> Colony colouration (MacConkey) or growth (MM) was determined after 24 h and labelled 3+ (very strong or good growth) to - (white or no growth). L, lethal.

<sup>b</sup> Growth was determined on sucrose MacConkey agar with and without cAMP (1 mM) and the same results obtained.

The results in Table 2.3 indicated that the *V. alginolyticus* sucrose utilization system was not expressed in the *E. coli crr* strain (JLV86). In addition, this lack of expression could not be relieved by the addition of cAMP to the growth medium. The *V. alginolyticus* sucrose utilization system was expressed in the *E. coli* parent strains (JWL314 and LR2-167). These results suggested that sucrose transport by *E. coli* strains transformed with the *V. alginolyticus* sucrose utilization system required EIII<sup>Glc</sup> *per se*. This result was consistent with the sequence data which suggested that the *V. alginolyticus* EII<sup>Scr</sup> protein was EIII-dependent and most likely interacted with EIII<sup>Glc</sup>. Since EII<sup>Glc</sup> and EIII<sup>Glc</sup> have been shown to be required for the transport of glucose (Postma and Lengeler, 1985), *E. coli* JLV86(pVS100) was unable to utilize glucose and the addition of cAMP did not relieve this deficiency. It has been shown that the EIII-like domains of EII<sup>Nag</sup> and EII<sup>Bgl</sup> were the functional equivalent of EIII<sup>Glc</sup> (Vogler *et al.*, 1988). Since *E. coli* JLV86 does not contain a functional EII<sup>Nag</sup> or EII<sup>Bgl</sup> protein the EIII-like domains of these proteins could not replace EIII<sup>Glc</sup> in EII<sup>Glc</sup>-dependent or EII<sup>Scr</sup>-dependent glucose or

sucrose transport, respectively. The PTS, through the action of EIII<sup>Glc</sup>, has been shown to activate adenylate cyclase. Cells lacking EIII<sup>Glc</sup> had low adenylate cyclase activity and as a consequence were unable to utilize a number of non-PTS carbon sources because expression of many operons were dependent on cAMP (Postma and Lengeler, 1985; Vogler *et al.*, 1988; Levy *et al.*, 1990). *E. coli* JLV86(pVS100) was unable to ferment rhamnose unless cAMP was supplied to the growth medium while lactose and glycerol were fermented more strongly when cAMP was supplied to the growth medium (Table 2.3). These results were indicative of the indirect effect of lowered cAMP levels due to the lack of EIII<sup>Glc</sup> rather than a requirement for EIII<sup>Glc</sup> *per se*.

**TABLE 2.3** Phenotypes of *crr* and parent strains of *E. coli* transformed with the *V. alginolyticus* sucrose utilization system on plasmid pVS100. Growth was determined on sucrose MM agar plates and MacConkey agar plates supplemented with various carbon sources.

Strain	Relevant Genotype	cAMP <sup>b</sup>	Phenotype <sup>a</sup>					
			MM	MacConkey				
			scr	scr	glc	lac	gly	rhm
JWL314 (pVS100)	<i>crr</i> <sup>+</sup> <i>nagE</i> <sup>+</sup>	+	ND	3+	3+	3+	3+	2+
		-	3+	3+	3+	3+	3+	2+
LR2-167 (pVS100)	<i>crr</i> <sup>+</sup> <i>nagE</i>	+	ND	3+	3+	3+	3+	2+
		-	3+	3+	3+	3+	3+	2+
JLV86 (pVS100)	<i>crr</i> <i>nagE</i>	+	ND	-	-	3+	3+	+
		-	-	-	-	+	+	-

<sup>a</sup> Colony colouration (MacConkey) or growth (MM) was determined after 24 h and labelled 3+ (very strong or good growth) to - (white or no growth). ND, not determined. Carbon sources tested were PTS sugars, sucrose (scr) and glucose (glc), class I sugars, lactose (lac) and glycerol(gly), and class II sugar rhamnose (rhm).

<sup>b</sup> This column indicates addition (+) or exclusion (-) of cAMP (1mM) to the growth medium.

**$\lambda$ ::Tn $phoA$  MUTAGENESIS:** Tn $phoA$  mutagenesis carried out on pVS103 resulted in the isolation of several recombinant plasmids conferring alkaline phosphatase activity on the PhoA<sup>-</sup> recipient *E. coli* CC118. The insertion points were restriction endonuclease mapped and those that mapped within the *V. alginolyticus* sucrose utilization system were selected for alkaline phosphatase activity determinations and sequence analysis. All the selected recombinant plasmids conferred high levels of alkaline phosphatase activity on the PhoA<sup>-</sup> recipient, *E. coli* CC118 (950 units/A<sub>600</sub>). DNA sequencing, employing a synthetic primer complementary to the *phoA* gene, was used to determine the exact point of insertion of the transposon. For all the recombinant plasmids selected, the point of insertion was identified to be in frame with the *scrA* gene at a point 454 bp downstream of the start of the gene (designated Tn $phoA\Delta 22$ ; Fig. 2.2). Tn $phoA\Delta 22$  appeared to be a hot-spot for insertion of the transposon into the *scrA* gene since no other alkaline phosphatase-positive insertion points were found out of those mapped within the *V. alginolyticus* sucrose utilization system. No alkaline phosphatase-positive insertions occurred into the *scrB* (also found by Scholle *et al.*, 1989) and *scrK* genes indicating that their gene products were not membrane-bound and did not have N-terminal signal sequences. The insertion into the *scrA* gene resulted in a predicted fusion protein with the alkaline phosphatase inserted 152 aa downstream from the N-terminus and within a hydrophilic region of the EII<sup>Scr</sup> protein. These results confirmed that the *V. alginolyticus* EII<sup>Scr</sup> protein was a membrane-bound protein and suggested that the region around aa residue 152 faced into the periplasm. It is very interesting that this region is close to a corresponding region in the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein predicted, on the basis of a hypothetical model, to face into the periplasm (Lengeler *et al.*, 1990; section 1.4.3 of the General Introduction).

### 2.3.2 Molecular analysis of the *V. alginolyticus scrK* gene

**NUCLEOTIDE SEQUENCE:** The *scrK* gene begins 151 bp downstream of the *scrA* TAA stop codon. The *scrK* gene consists of a presumptive ATG start codon and a TGA stop codon, and contains 921 bp which encodes a polypeptide of 307 aa residues (Fig. 2.2). The  $M_r$  of the predicted polypeptide is 33 045.

The ATG start codon is not preceded by a classical ribosome-binding site (Shine and Dalgarno, 1974). An AAGG sequence is located 8 bp upstream of the ATG start codon. A promoter-like sequence (Hawley and McClure, 1983) is located upstream of the ATG start codon (TTGGCT-N<sub>14</sub>-TTTACT; Fig. 2.2). The TGA termination codon is located 31 bp upstream of the ATG start codon for the *scrB* gene. No potential Rho-independent transcriptional termination loop is located in the intergenic region.

**DEDUCED AA SEQUENCE AND HOMOLOGY COMPARISONS:** The *V. alginolyticus* *scrK* gene product was found to be homologous to the *E. coli* enzymes, 6-phosphofructokinase, isoenzyme 2 (gene *pfkB*; Daldal, 1984) and ribokinase (gene *rbsK*; Hope *et al.*, 1986). The predicted aa sequence was aligned against these enzymes and the % similarity, with respect to identical or identical and conservatively changed aa residues (in parenthesis), was 19% (29%) for 6-phosphofructokinase and 18% (29%) for ribokinase. This result suggested that the *V. alginolyticus* *scrK* gene encoded a fructokinase. A fructokinase has been implicated in the *S. typhimurium* (pUR400 system) sucrose utilization system (Schmid *et al.*, 1988) and the chromosomally encoded sucrose utilization system from *K. pneumoniae* (Sprenger and Lengeler, 1988).

The aa sequence comparisons revealed two regions of relatively high homology; one at the N-terminus and the other at the C-terminus (Fig. 2.6). A common nucleotide binding fold has been identified in ATP synthase, myosin, kinases and other ATP-requiring enzymes (Walker *et al.*, 1982). The conserved aa sequences proposed to contribute to the nucleotide binding fold, G-X<sub>4</sub>-GK(T)-X<sub>6</sub>-I/V and R/K-X<sub>3</sub>-G-X<sub>3</sub>-L-(Hydrophobic)<sub>4</sub>-D, were not homologous to the regions of high homology presented in Fig. 2.6 and were not present in the remainder of the *V. alginolyticus* fructokinase aa sequence. Thus, the functional significance of the regions presented in Fig. 2.6 remains to be resolved.

## N-TERMINAL REGION

ScrK	27	G	G	A	P	A	N	V	A	V	A	I	A	R	L	S	G	-	K	S	A	F	F	G	R	V	G	D	D	P	F	G	56
RbsK	41	G	G	K	G	A	N	Q	A	V	A	A	G	R	-	S	G	A	N	I	A	F	I	A	C	T	G	D	D	S	I	G	70
PfkB	38	G	G	G	-	I	N	V	A	R	A	I	A	H	L	G	G	S	A	T	A	I	F	P	A	-	G	G	A	T	-	G	65

## C-TERMINAL REGION

ScrK	236	G	Q	V	V	S	P	I	D	T	T	G	A	G	D	A	F	V	G	G	L	L	A	C	L	259
Rbsk	242	G	F	R	V	Q	A	V	D	T	I	A	A	G	D	T	F	N	G	A	L	I	T	A	L	265
PfkB	242	P	P	A	L	K	S	Q	S	T	V	G	A	G	D	R	L	V	G	A	M	T	L	K	L	265

**FIG. 2.6** Two regions of high homology found when comparing the aa sequence of the *V. alginolyticus* fructokinase (ScrK) to those of the *E. coli* enzymes ribokinase (RbsK) and 6-phosphofructokinase (isoenzyme 2) (PfkB). The aa are identified by the single-letter code and identical aa are boxed. The positions of the aa in the enzymes are indicated at the beginning and end of each sequence.

**ENZYME ACTIVITY STUDIES:** The results of the enzyme activity studies confirmed that the *V. alginolyticus* *scrK* gene encoded a fructokinase. Fructokinase activity was high in sucrose grown cultures and low in glucose or fructose grown cultures indicating that *scrK* gene expression was regulated in *E. coli* JA221(pVS100) (Table 2.4). Similarly, expression of the *scrA* and *scrB* genes was regulated. Control cultures of *E. coli* JA221 had no detectable fructokinase activity indicating that this fructokinase activity was not due to an enzyme (mannofructokinase, for example) produced by *E. coli* JA221 (Table 2.4).

The 1.7-kb *EcoRV-EcoRV* restriction endonuclease fragment of pVS104 was subcloned into Bluescript SK to give plasmid pVS115 (Fig. 2.1). In *E. coli* JA221(pVS115) *scrK* expression was no longer regulated; *scrK* expression was constitutive and fructokinase activity was high in glucose grown cultures (Table 2.4). These results obtained with plasmid pVS115 confirmed that the *scrK* gene resided on the 1.7-kb *EcoRV-EcoRV* restriction endonuclease fragment of pVS104 and was therefore located between the *scrA* and *scrB* genes.

**TABLE 2.4** Fructokinase, sucrase, and sucrose transport specific activities for *E. coli* JA221 and *E. coli* JA221 transformants after growth in MM supplemented with various carbon sources.

Strain	Carbon Source <sup>a</sup>	Enzyme Specific Activity <sup>c</sup>		
		Fructokinase	Sucrase	Sucrose transport
JA221(pVS100)	scr	12.91	6.86	2.29
	glc	0.85	0.28	0.29
	fru	0.00	0.13	ND
JA221	scr <sup>b</sup>	0.00	0.00	0.00
	glc	0.00	0.00	0.00
	fru	0.00	0.00	ND
JA221(pVS115)	glc	13.01	0.00	ND
JA221(SK)	glc	0.00	0.00	ND

<sup>a</sup> The cells were grown to the late-exponential phase ( $A_{600}=1.0$ ) on MM supplemented with sucrose (scr), glucose (glc) or fructose (fru).

<sup>b</sup> The cells were grown on sucrose plus glycerol (1 %, v/v).

<sup>c</sup> Fructokinase specific activity was expressed as nmoles phosphorylated fructose per min per mg protein. Sucrase specific activity was expressed as  $\mu$ moles reducing sugar per min per mg protein. Sucrose transport specific activity was expressed as nmoles [<sup>14</sup>C]sucrose per min per mg protein.

### 2.3.3 DNA-directed cell-free protein synthesis

Cell-free coupled transcription-translation of pVS103 (entire sucrose utilization system) resulted in the production of three major proteins with apparent  $M_r$  of approximately 59 600, 51 300 and 34 700 (Fig. 2.7). These proteins were not produced by the vector pEcoR251 (Fig. 2.7). These apparent  $M_r$  were similar to those obtained for plasmid pVS100 (Scholle, 1989) and seemingly corresponded to the predicted  $M_r$  for the *scrB*, *scrA* and *scrK* gene products (55 657, 49 889, and 33 045, respectively). This was confirmed by analyzing various subclones of pVS103 (Fig. 2.7). Cell-free coupled transcription-translation of plasmids pVS114 (*scrA* gene) and pVS115 (*scrK* gene) resulted in the production of major proteins (not produced

by the vector Bluescript SK) of apparent  $M_r$  of approximately 51 300 and 34 700, respectively (Fig. 2.7). The two polypeptides of apparent  $M_r$  of approximately 51 300 and 34 700 therefore corresponded to the  $EII^{Scr}$  protein and the fructokinase, respectively. Cell-free coupled transcription-translation of plasmid pVS110 (*scrB* and *scrK* genes) resulted in 2 major proteins (not produced by the vector Bluescript SK) of apparent  $M_r$  of approximately 59 600 and 34 700 (Fig. 2.7). The polypeptide of apparent  $M_r$  of approximately 59 600 therefore corresponded to the sucrase enzyme.

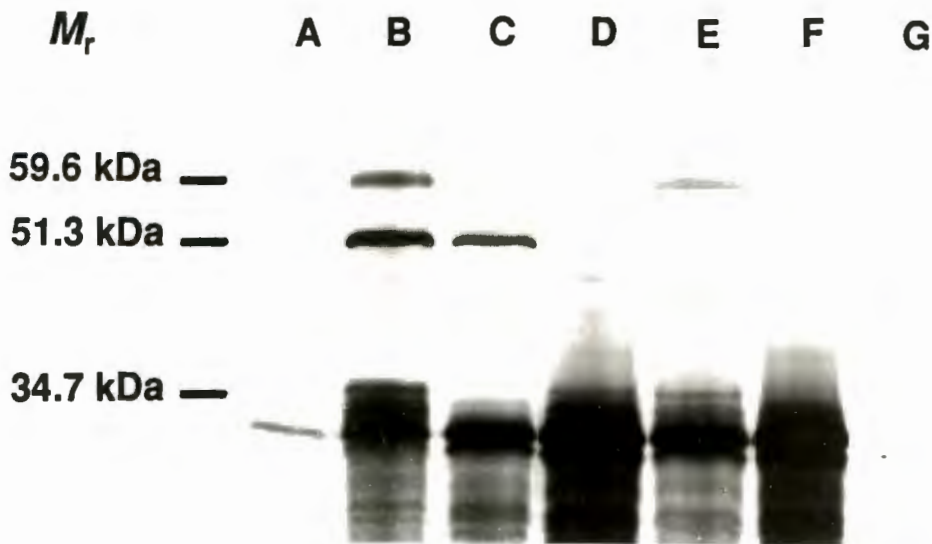


FIG. 2.7 Autoradiogram of the cell-free coupled transcription-translation polyacrylamide gel. Lanes A-F: Translation products of *in vitro* transcribed and translated plasmids. Lane A: pEcoR251, Lane B: pVS103, Lane C: pVS114, Lane D: pVS115, Lane E: pVS110, Lane F: Bluescript SK. Lane G: Zero DNA control.

### 2.3.4 Codon usage in 15 *V. alginolyticus* genes

The codon usage in the *V. alginolyticus* *scrA* and *scrK* genes was compared to that of 13 other *V. alginolyticus* genes (Table 2.5). The codon frequency (per 1000) for all 15 *V. alginolyticus* genes was calculated and compared to codon frequencies published for 941 *E. coli* genes, 150 *B. subtilis* genes and 99 *S. typhimurium* genes (Table 2.5).

TABLE 2.5 Codon usage for 15 *V. alginolyticus* genes compared with that for 941 *E. coli* (*Ec*) genes, 150 *B. subtilis* (*Bs*) genes and 99 *S. typhimurium* (*St*) genes.

Residue and codon	<i>V. alginolyticus</i> genes <sup>a</sup>							<i>Ec</i> <sup>b</sup> <i>Bs</i> <sup>b</sup> <i>St</i> <sup>b</sup>				
	<i>scrA</i>	<i>scrB</i>	<i>scrK</i>	<i>proA</i>	<i>glnA</i>	<i>ntxB</i>	<i>unc operon</i>	T <sub>1</sub>	T <sub>2</sub>	T <sub>2</sub>	T <sub>2</sub>	T <sub>2</sub>
Phe UUU	17	22	13	11	7	2	28	100	21	19	26	20
Phe UUC	10	7	4	4	19	2	54	100	21	18	14	15
Leu UUA	11	6	5	8	1	8	17	56	12	10	19	12
Leu UUG	17	17	5	8	0	12	24	83	17	11	13	12
Leu CUU	11	11	3	6	10	8	48	97	20	10	24	10
Leu CUC	1	4	3	5	0	7	6	26	5	10	10	10
Leu CUA	6	3	3	6	16	3	89	126	26	3	5	4
Leu CUG	11	6	5	3	2	11	39	77	16	55	21	56
Ile AUU	24	11	8	8	3	16	53	123	25	27	35	28
Ile AUC	16	15	3	9	23	12	98	176	36	28	27	27
Ile AUA	4	2	3	3	0	1	4	17	4	4	8	4
Met AUG	15	13	10	10	19	8	74	149	31	27	27	26
Val GUU	19	3	9	10	16	7	72	136	28	21	19	15
Val GUC	6	8	7	11	0	6	9	47	10	14	17	19
Val GUA	5	3	4	11	13	1	80	117	24	12	15	12
Val GUG	8	10	9	17	5	5	28	82	17	25	17	26
Ser UCU	8	7	7	10	16	5	42	95	20	11	15	9
Ser UCC	3	1	0	5	0	0	4	13	3	10	9	12
Ser UCA	4	4	5	7	11	2	24	57	12	6	15	6
Ser UCG	1	5	2	13	3	3	19	46	10	8	6	9
Ser AGU	2	9	5	16	0	6	7	45	9	7	6	6
Ser AGC	4	5	6	17	2	6	17	57	12	15	14	17
Pro CCU	4	11	3	5	6	2	28	59	12	6	10	8
Pro CCC	1	0	1	2	0	3	2	9	2	4	3	5
Pro CCA	10	11	3	10	16	8	40	98	20	8	7	6
Pro CCG	6	0	4	5	2	5	10	32	7	24	15	26
Thr ACU	5	7	4	5	6	5	36	68	14	11	9	8
Thr ACC	5	6	3	9	1	5	13	42	9	24	8	26
Thr ACA	5	6	7	8	10	6	30	72	15	6	24	5
Thr ACG	9	8	4	8	4	4	22	59	12	12	14	17
Ala GCU	9	4	9	10	16	4	78	130	27	18	19	14
Ala GCC	5	3	4	8	0	5	21	46	10	23	13	28
Ala GCA	21	6	9	17	22	4	87	166	34	20	23	11
Ala GCG	27	3	10	17	9	11	60	137	28	33	19	40
Tyr UAU	4	13	0	13	0	4	13	47	10	15	21	15
Tyr UAC	8	7	0	9	16	1	50	91	19	14	12	12
His CAU	2	12	3	3	0	5	13	38	8	11	15	11
His CAC	2	8	0	4	13	6	18	51	11	11	8	9
Gln CAA	10	13	17	25	7	24	49	145	30	13	22	12
Gln CAG	4	12	3	2	2	7	46	76	16	30	19	33
Asn AAU	5	8	6	10	0	6	14	49	10	16	23	18
Asn AAC	16	13	2	17	17	6	56	127	26	25	21	23
Lys AAA	16	17	8	7	18	9	79	154	32	37	53	36
Lys AAG	8	10	4	4	7	5	41	79	16	12	20	12
Asp GAU	10	17	13	20	10	10	53	133	27	32	34	34
Asp GAC	5	17	7	19	28	9	59	144	30	23	22	22
Glu GAA	7	14	10	5	27	14	99	176	36	44	53	41
Glu GAG	5	17	7	8	7	7	65	116	24	20	24	22
Cys UGU	1	4	3	9	2	1	10	30	6	5	3	4
Cys UGC	1	1	2	2	1	0	5	12	2	6	4	5
Trp UGG	3	9	6	5	2	2	14	41	8	13	9	11
Arg CGU	3	8	3	5	13	7	67	106	22	26	9	23
Arg CGC	3	2	3	6	6	7	28	55	11	22	9	25
Arg CGA	0	3	2	3	0	3	4	15	3	3	4	3
Arg CCG	0	0	1	0	0	0	0	1	0	4	6	5
Arg AGA	0	4	2	3	0	2	3	14	3	2	12	2
Arg AGG	0	1	0	0	0	0	1	2	0	1	4	1
Gly GGU	32	15	15	25	25	5	131	248	51	29	14	19
Gly GGC	15	12	4	16	9	7	31	94	19	31	24	35
Gly GGA	4	3	4	7	0	6	9	33	7	7	22	6
Gly GGG	5	7	2	5	0	2	3	24	5	9	9	10

LEGEND: SEE NEXT PAGE

## LEGEND TO TABLE 2.5

<sup>a</sup> The *V. alginolyticus* genes listed are the sucrose uptake-encoding gene (*scrA*), the sucrose-encoding gene (*scrB*; Scholle *et al.*, 1989), the fructokinase-encoding gene (*scrK*), the protease-encoding gene (*proA*; Deane *et al.*, 1989), the glutamine synthetase-encoding gene (*glnA*; Maharaj *et al.*, 1989), the NR<sub>II</sub>-encoding gene (*ntrB*; Maharaj *et al.*, 1989) and a compilation of 9 genes (*uncA-uncI*) involved in the *unc* operon for ATP synthase (Krumholz *et al.*, 1989). The numbers listed for each gene represent the number of times each codon is used. T<sub>1</sub> lists the total number of times a codon is used for all the genes. T<sub>2</sub> lists the frequency (per 1000) at which each codon is used.

<sup>b</sup> These codon frequency tables were reported by Wada *et al.* (1990).

The codon usage in the *V. alginolyticus* *scrA* and *scrK* genes reflected the bias of the other *V. alginolyticus* genes. The codon frequency table for all 15 *V. alginolyticus* genes was a stronger reflection of the bias observed in the *E. coli* and *S. typhimurium* codon frequency tables than the bias observed in the *B. subtilis* codon frequency table. The preferred codon usages reported for *E. coli* (Konigsberg and Godson, 1983) for leucine (CUG), proline (CCG) and glutamine (CAG) were observed for genes from *S. typhimurium* but not for genes from *V. alginolyticus* or *B. subtilis*. For the *V. alginolyticus* genes leucine was encoded at comparable frequencies by 5 of the 6 possible codons, proline showed preference for CCU and CCA codons, and glutamine was preferentially encoded by CAA. For the *B. subtilis* genes leucine was encoded at comparable frequencies by 5 of the 6 possible codons (different 5 to *V. alginolyticus*), proline showed preference for CCU and CCG codons and glutamine was encoded at equal frequencies by both possible codons. While *V. alginolyticus*, *E. coli* and *S. typhimurium* genes showed similar codon usages for arginine and glycine, *B. subtilis* genes differed in their codon usage for these aa (Table 2.5).

## 2.4 CONCLUSION

The *V. alginolyticus* sucrose uptake-encoding region was sequenced and contained an *scrA* gene encoding a sucrose uptake protein, EII<sup>Scr</sup>, and an *scrK* gene encoding a fructokinase. The promoter-like sequence located upstream of the start of the *scrA* gene was very similar to the promoter consensus sequence derived by Hawley and McClure (1983). The promoter-like sequence located in the intergenic region between the *scrA* and *scrK* genes was less well conserved, while no promoter-like sequence was located in the intergenic region between the *scrK* and *scrB* genes. It is therefore possible that the *scrA*, *scrK*, and *scrB* genes form an operon transcribed and regulated from the promoter region upstream of the *scrA* gene. Two inverted repeats which may act as Rho-independent transcriptional terminators have been located; the first in the intergenic region between the *scrA* and *scrK* genes and the second downstream of the *scrB* gene (Scholle *et al.*, 1989). The latter was more likely to function as a Rho-independent transcriptional terminator since it was situated at the end of the operon.

Sucrase production by *V. alginolyticus* and *E. coli* (pVS100) was shown to be subject to glucose repression which was relieved by cAMP (Scholle *et al.*, 1987). In addition, studies with *crp* and *crp cya* mutants of *E. coli* suggested that a functional CAP was required for expression of the *V. alginolyticus* sucrose utilization system in *E. coli* (Scholle, 1989). It was suggested that a CAP binding site was most likely located in a promoter region upstream of the *scrA* gene since no CAP binding site was found after sequencing the *scrB* gene and its flanking regions (Scholle, 1989; Scholle *et al.*, 1989). However, no sequences significantly similar to the CAP binding site consensus sequence (Berg and von Hippel, 1988) were found in the promoter regions upstream of the *scrA* gene or the *scrK* gene. The mechanism by which CAP is involved in the expression of the *V. alginolyticus* sucrose utilization system remains to be resolved. While glucose repression of the *V. alginolyticus* sucrose utilization system appears to occur at the level of enzyme synthesis (involving CAP), glucose repression also needs to be considered at the level of enzyme activity. Since the *V. alginolyticus* sucrose transport protein is most likely an EIII<sup>Glc</sup>-dependent EII<sup>Scr</sup> of the PTS, growth of *E. coli* (pVS100) in the presence of

both sucrose and glucose will result in competition between EII<sup>Glc</sup> and EII<sup>Scr</sup> for EIII<sup>Glc</sup> and the general PTS components, HPr and EI. Since EIII<sup>Glc</sup> is more specific for EII<sup>Glc</sup>, growth of *E. coli* (pVS100) on both glucose and sucrose will result in the preferential use of glucose. Under these growth conditions, therefore, the activity of the *V. alginolyticus* EII<sup>Scr</sup> protein is reduced due the unavailability of EIII<sup>Glc</sup> as well as competition for the general PTS components. As a result, less sucrose is transported into the cells (inducer exclusion) and the *V. alginolyticus* sucrose utilization system is not fully induced. Therefore, glucose repression of the *V. alginolyticus* sucrose utilization system may occur both at the level of enzyme activity (involving the PTS) and enzyme synthesis (involving CAP).

This is the first report of the nt and deduced aa sequence of a fructokinase-encoding gene associated with a sucrose utilization system. It will be very interesting to compare the aa sequence of the *V. alginolyticus* fructokinase to those of *S. typhimurium* (pUR400 system) and *K. pneumoniae* when their sequences become available. While the *V. alginolyticus* sucrose enzyme showed relatively high identical aa similarity to the *B. subtilis* sucrose (34%) and lower identical aa similarity to the *S. mutans* sucrose (21%) (Scholle *et al.*, 1989), the situation for the EII<sup>Scr</sup> proteins was reversed. The *V. alginolyticus* EII<sup>Scr</sup> protein had relatively high identical aa similarity to the *S. mutans* EII<sup>Scr</sup> protein (46%) and lower identical aa similarity to the *B. subtilis* EII<sup>Scr</sup> protein (38%). However, while the *S. mutans* EII<sup>Scr</sup> protein contained an EIII-like domain and was EIII-independent (Sato *et al.*, 1989), the *V. alginolyticus* EII<sup>Scr</sup> protein like the *S. typhimurium* (pUR400 system) EII<sup>Scr</sup> protein (Lengeler *et al.*, 1982) was EIII<sup>Glc</sup>-dependent. The *V. alginolyticus* sucrose utilization system resembled the *S. typhimurium* (pUR400) system which also had the *scrK* and *scrA* genes situated upstream of the *scrB* gene within a single operon, and transcribed in the same direction. However, unlike the *S. typhimurium* (pUR400) sucrose utilization system, the *V. alginolyticus* system did not contain an *scrY* gene encoding a sucrose porin.

## CHAPTER THREE

NUCLEOTIDE SEQUENCE AND ANALYSIS OF THE  
*V. ALGINOLYTICUS* SCR REPRESSOR-ENCODING GENE (*scrR*)

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## CHAPTER THREE

### NUCLEOTIDE SEQUENCE AND ANALYSIS OF THE *V. ALGINOLYTICUS* SCR REPRESSOR-ENCODING GENE (*scrR*)

**SUMMARY:** Nucleotide sequencing of the region upstream of the *V. alginolyticus* *scrA* gene located an Scr repressor-encoding gene (*scrR*) and an unidentified ORF (ORF5). Both genes were in the opposite orientation with respect to the *scrA* gene. The deduced aa sequence of the Scr repressor was homologous to the Gal, Lac and Cyt repressors of *E. coli* and contained a helix-turn-helix DNA binding domain. The codon usage of the *scrR* gene and ORF5 did not reflect the bias observed for 15 other *V. alginolyticus* genes. The *scrR* gene was shown to produce a protein in a DNA-directed cell-free coupled transcription-translation system. The *scrR* gene was required *in cis* for regulation of the *V. alginolyticus* sucrose utilization system in *E. coli*, and a deletion of the *scrR* gene could not be complemented *in trans*.

### 3.1 INTRODUCTION

All bacterial sucrose utilization systems studied to date have been shown to involve regulatory proteins and the various regulatory processes have been reviewed in the General Introduction (section 1.4). In *B. subtilis* the synthesis of levansucrase (gene *sacB*) is regulated by the *sacY* gene product ( $M_r$  32 456) and *sacX* gene product ( $M_r$  49 024). The *sacY* gene product appears to be a positive regulator which acts as an antiterminator relieving termination of *sacB* expression at the *sacR* locus (Crutz *et al.*, 1990). The *sacX* gene product, in conjunction with the PTS, appears to negatively regulate the activity of the *sacY* gene product. The synthesis of the *B. subtilis* sucrose transport protein (EII<sup>Scr</sup>) and sucrose enzyme (*sacPA* operon) appears to be under the control of the *sacT* gene product ( $M_r$  32 037) and may also involve antitermination, however, this remains to be investigated (Debarbouille *et al.*, 1990). In *S. typhimurium* (pUR400 system) a single gene (*scrR*) encoding a repressor has been found to be involved in sucrose metabolism (Schmid *et al.*, 1988). The pUR400-encoded structural genes, *scrK*, *scrY*, *scrA* and *scrB*, were shown to be under the control of the *scrR* gene product ( $M_r$  37 000). In *scrR*<sup>+</sup> cells expression of the four structural genes was inducible, but when *scrR* was deleted expression of these genes was constitutive. The chromosomally encoded sucrose utilization system of *K. pneumoniae* is similar to that of *S. typhimurium* (pUR400 system) and also involves an *scrR* gene encoding a repressor. In *S. mutans* sucrose transport by an EII<sup>Scr</sup> of the PTS (gene *scrA*) was found to be inducible while sucrose-6-phosphate hydrolase activity (gene *scrB*) was constitutive (St. Martin and Wittenberger, 1979b). This result was compatible with nt sequencing data which showed that the *scrA* gene was located immediately upstream of the *scrB* gene and divergently transcribed from the opposite DNA strand (Sato *et al.*, 1989). For *S. mutans* two genes encoding regulatory proteins have been located downstream of the *scrB* gene, however, these genes remain to be investigated (Sato *et al.*, 1989).

The synthesis of the *V. alginolyticus* sucrose enzyme and sucrose transport protein (EII<sup>Scr</sup>) was shown to be inducible by sucrose (Scholle *et al.*, 1987; section 1.4.4 of the General Introduction). In addition, synthesis of the *V. alginolyticus* fructokinase also

appeared to be inducible by sucrose (section 2.3.2). Therefore, the *V. alginolyticus* sucrose utilization system may involve a gene(s) encoding a regulatory protein(s). To locate and characterize the gene(s) encoding the *V. alginolyticus* regulatory protein(s) involved in sucrose metabolism, the DNA upstream of the *scrA* gene was sequenced and characterized.

## 3.2 MATERIALS AND METHODS

### **3.2.1 Bacterial strains and plasmids**

*E. coli* JA221 (Table 2.1) transformants were used to study sucrose production while *E. coli* LK111 served as a recipient for exonuclease III shortened plasmids. The construction of plasmid pVS103 was described in section 2.2.3. Plasmids pVS116, pVS117, pVS118, pVS119, pVS120 and pVS121 were constructed in this study. The M13-derived Bluescript SK plasmid (Stratagene, San Diego) was used for the preparation of nt sequencing templates (Appendix B). Plasmid pACYC177 (Chang and Cohen, 1978) which is compatible with *ColE1*- or *pMB1*-derived plasmids, was used to construct plasmids for the *in trans* studies (Appendix B).

### **3.2.2 Standard methods, media, buffers and solutions**

Standard methods, media, buffers and solutions are described in Appendix A.

### **3.2.3 Subcloning the sucrose utilization system**

The 1.3-kb *EcoRI*(vector)-*SacI* and 1.7-kb *EcoRI*(vector)-*BglIII* restriction endonuclease fragments of pVS103 were deleted and the resulting 8.2-kb and 7.8-kb restriction endonuclease fragments were self-ligated to generate pVS116 and pVS117, respectively (Fig. 3.1). Prior to ligation, the sticky ends generated by restriction endonuclease digestion were filled in to allow recircularization by blunt end self-ligation. Restriction endonuclease fragments to be filled in, were purified by one phenol extraction and several ether extractions, followed by an ethanol precipitation in the presence of 0.3 M sodium acetate (Appendix A). Filling-in reactions were carried out in T4 Polymerase buffer (Appendix A) in the presence of 1 unit/ $\mu$ g DNA of T4 Polymerase enzyme (Boehringer Mannheim) and 250  $\mu$ M of each dNTP. Reactions were allowed to proceed for 10 min at 37°C before being terminated by another purification step.

Plasmid pVS118 was derived by subcloning the 5.3-kb *Bgl*III-*Pst*I(vector) restriction endonuclease fragment of pVS103 into *Bam*HI-*Pst*I restriction endonuclease digested pACYC177 (Fig. 3.1). Plasmids pVS119 and pVS120 were derived by subcloning the 1.5-kb *Hind*III-*Hpa*I and 1.1-kb *Hind*III-*Bgl*III restriction endonuclease fragments of pVS103 into *Hind*III-*Eco*RV and *Hind*III-*Bam*HI restriction endonuclease digested Bluescript SK, respectively (Fig. 3.1). Plasmid pVS121 represented an exonuclease III shortened version (shortened from the *Hind*III end) of pVS119 which contained a 0.6-kb insert (Fig. 3.1). *E. coli* was transformed as described in Appendix A. Transformants were selected on LB or sucrose MacConkey agar containing Ap (100 µg/ml) (Appendix A). All other details necessary for subcloning have been mentioned in section 2.2.3.

#### 3.2.4 Exonuclease III digestion

Exonuclease III deletions of pVS119 were generated according to a modification of the method of Henikoff (1984). Progressive deletions from the 5' end of the insert were generated by unidirectionally digesting *Apa*I-*Cla*I restriction endonuclease digested pVS119 (Henikoff, 1984). Similarly, deletions were made from the 3' end of the insert using *Bst*XI-*Xba*I restriction endonuclease digested pVS119. The experimental details for exonuclease III digestion and the selection of suitable DNA sequencing templates were described in section 2.2.4.

#### 3.2.5 Nucleotide sequencing

The nt sequence of both strands of the DNA upstream of the *scrA* gene was determined using the overlapping DNA fragments generated by exonuclease III digestion of pVS119. DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method described in Appendix A.

The nt and deduced aa sequences were analyzed on an IBM XT computer using the Genepro programme version 4.1 (Riverside Scientific) and the GCG Package version 6.1. All the current databases accompanying the GCG Package were screened for related nt and aa sequences.

### 3.2.6 Sucrase assays

Sucrase assays were carried out on late-exponential-phase cultures of *E. coli* transformants ( $A_{600}=1.0$ ). Samples were assayed in duplicate and experiments were repeated three times.

Cells (20 ml) were grown at 37°C in MM supplemented with sucrose or glucose and Ap (50 µg/ml) (Appendix A). Cell extracts were prepared from these cells as described in section 2.2.7. Sucrase assays were performed on the cell extracts by a modification of the method described by Scholle *et al.* (1987) as described in section 2.2.7. Sucrase specific activity was expressed as µmoles of reducing sugar produced per min per mg protein.

### 3.2.7 Protein assays

Protein concentrations were determined by the method of Bradford (1976) as described in section 2.2.8.

### 3.2.8 DNA-directed cell-free protein synthesis

The synthesis of proteins by plasmids pVS119, pVS120, and pVS121 was investigated in an *E. coli* DNA-directed cell-free system (Amersham, Prokaryotic DNA-directed cell-free translation kit, Code N.380). The experimental procedure was similar to that described in section 2.2.10. The labelled proteins were separated on 20% SDS-PAGE gels (Laemmli, 1970; O'Farrell, 1975) so that low  $M_r$  proteins could be resolved (Appendix A). Insulin ( $M_r$  6000; sigma) was used in addition to the low molecular weight standards described in section 2.2.10.

### 3.3 RESULTS AND DISCUSSION

Since nucleotide sequencing and analysis of the sucrose uptake-encoding region did not reveal any genes encoding regulatory proteins, the DNA upstream of the *scrA* gene was sequenced. DNA sequencing templates generated by exonuclease III digestion from the 5' and 3' ends of the insert in plasmid pVS119 were constructed, and overlapping sequence data were obtained for both DNA strands of the insert (Fig. 3.1 and 3.2).

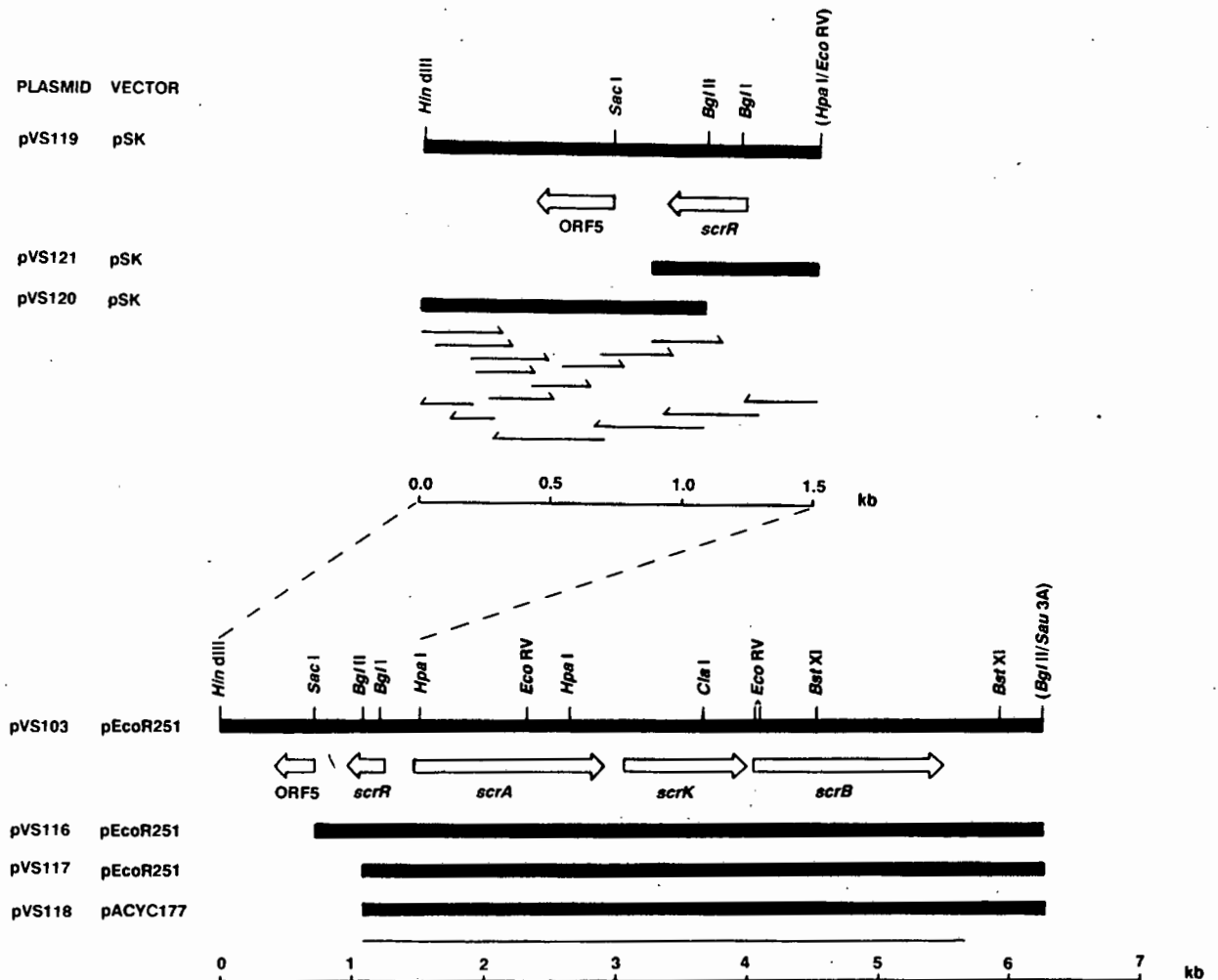


FIG. 3.1 Restriction map of pVS103 insert DNA, subclones derived from pVS103 and the subcloning and sequencing strategy for the *V. alginolyticus* *scrR* gene and ORF5 on pVS119. The bold lines, single thin line and open arrows represent *V. alginolyticus* insert DNA, the extent of sequencing previously reported (section 2.3) and the ORF's, respectively. The thin arrows represent the extent and direction of sequencing of templates generated by exonuclease III digestion.

The region of overlap with the upstream region of the *scrA* gene was identified. Two ORF's were present upstream of the *scrA* gene on the opposite DNA strand (Figs 3.1 and 3.2). On the basis of homology with other repressor genes, the ORF adjacent to the *scrA* gene was identified as the putative *scrR* gene coding for an Scr repressor while the second ORF remained unidentified (ORF5).

```

1  TCA ATT GTT GAG AAA ACG ATG ACG GCC TAT TAA GTA
12 T  L  L  E  K  A  V  A  P  Y  N  fM
                                     ← scrA
                                     TTCATACCCCATGGATTATTATCTGAGCTTG
69 CAGAATTTTCGGGAACGTTTGC-35AAACAGATTTTGTCAATAAAACCAAATGATCAAAGATAAAGCAGAGAGAATGTGA
148 GAATGATCGTCAAATTTCTCCCTAAGAGCAGTATAGGAGGT-10TAGAAATCGGCTACTTATCAAAGAGCCTTTCATCTTCTC
227 TTTGCAAACAATCCCAAAAACGAAAAACAGAATGGATSDAGGAACA  ATG GCG AGT CTG CAT GAT GTC GCC
1  fM A S L H D V A
                                     scrR →
295 CGT CTG GCT GGC GTA TCA AAA TCA ACA GTG TCA CGG GTT ATA AAC GAT GAG TAT GGC GTA
9  R  L  A  G  V  S  K  S  T  V  S  R  V  I  N  D  E  Y  G  V
355 AAA GAA GCG ACA AAA CAG AAG GTC CGC CAA GCA GTA GCG GAG TGT GGC TAT GTG CCG AAC
29 K  E  A  T  K  Q  K  V  R  Q  A  V  A  E  C  G  Y  V  P  N
415 CAA GTC GCT AAA GAT CTT AAA GAA GAA AGC TCT GCC ATT ATT GTT CAA GAC AAA ACA GGC
49 Q  V  A  K  D  L  K  E  E  S  S  A  I  I  V  Q  D  K  T  G
475 AAA GTG CTG TTT GTA AAA GAA GGC GCA CTT GAG CAA GAT GAA ATT GCA AAG GTG ATC GAG
69 K  V  L  F  V  K  E  G  A  L  E  Q  D  E  I  A  K  V  I  E
535 CTA ATT AAA CAG AAC ATC TAG  TCTCGTTGCTGGCTCCTTAATATCCGTAATACACAATATAAGGAGCC
89 L  I  K  Q  N  I  *
604 ATGCGACCAAACCTAAAGCTGAGATTCTGTGCCCCTAGTTGAACGCTTAGTGATTCCACCAAGGAGACTGTCTCAGA
683 TTCGGCGACATCACATGTGTTGACGTAAATTTCCCACTAGCCAACAAAAGAGTGAAC-35TCTCATTATGGGTGTTA
762 -10TATTATAACAGTCGGTAASDGAGGAGCTCAG  ATG TAC CAC CAC CAG CAA AAG ATA CGG AAG CAT TGG
1  fM Y H H Q Q K I R K H W
                                     ORF5 →
827 CAT CGC ACT GTT TTA TTT TTC AGT GTC GCG TTG CTG ATC GCT TGG AAC TTT GCG GTA ATC
13 H  R  T  V  L  F  F  S  V  A  L  L  I  A  W  N  F  A  V  I
887 CTT CAT CAA GTT GAT CTG ACT CCC GAA CAC CAC ACA CAC CAT CAT TGC CAG CTA TTT TCT
33 L  H  Q  V  D  L  T  P  E  H  H  T  H  H  H  C  Q  L  F  S
947 GGG GTT CAG CAC GGC ATA GCC AAA GCT CAA CCG ACC CTA TCG ACG CCA ACA TTT ACG CGC
53 G  V  Q  H  G  I  A  K  A  Q  P  T  L  S  T  P  T  F  T  R
1007 ATC CAA TAC CAT GAT GTC TTT CAG CGC CTT GTT AAT AGT GAA GAC ATT CGT GGT GCA GCT
73 I  Q  Y  H  D  V  F  Q  R  L  V  N  S  E  D  I  R  G  A  A
1067 CGT GCC CCG CCT TAT TTT GCT TAA  TTTCCAAACTATTCCCACTCAACATAATTTGTGCGTATTCTCTGCA
93 R  A  P  P  Y  F  A  *
1137 CCTCACGTGTCAGCGAATCGCACCTTAGTAAATACAAATCTGATCCGGTATTGCTCGGGTTAGTGCCAGCAAATAGG
1216 AAACACAAAATGTCATCGAAACATATTTTAGCGCTGTTCATTGGTCTCTTTATCGACAGCAGCAAATGCGGACGAAT
1295 ACCGCCAGCACGGTGCATGTGCATGGTCACGTCAATTTAATATCGCCCAAGATGGAAAGGATCTGTTAGTGGAGAT
1374 TACTGCTCCCGGTGCCGATGTGGTTCGGTTTCGAGCATGCTCCAGAAAATGAACAACAAGCAAGCAACTTAAACAAGCC
1453 ATTGCCACCCTAGAAGACAGCAACACACTGTTTGGGATTAACGCGCAAGCCAATTGTGATATTGAAGAAGCT

```

FIG. 3.2 Nucleotide sequence upstream of the *scrA* gene containing the *scrR* gene and ORF5 from *V. alginolyticus* (GenBank accession number: M35009). The deduced aa sequences are shown in single-letter code below the coding sequences. The nt sequence is numbered throughout while the aa sequences of the *scrR* and ORF5 gene products are numbered separately. Putative promoters (-35 and -10 regions) and the ribosome-binding sites (SD) are in boldface type and underlined. The putative Scr repressor binding sites homologous to the *gal* operator are underlined. The opposite DNA strand coding for the first 12 aa of the *scrA* gene product is shown at the beginning of the sequence.

### 3.3.1 Molecular analysis of the *V. alginolyticus scrR* gene

**NUCLEOTIDE SEQUENCE:** The *scrR* gene lies in the opposite orientation with respect to the *scrA* gene. From the presumptive ATG start codon to the presumptive TAG stop codon the *scrR* gene contains 282 bp which encode a polypeptide of 94 aa residues (Fig. 3.2). The  $M_r$  of the predicted polypeptide is 10 268. The ATG start codon is not preceded by a classical ribosome-binding site (Shine and Dalgarno, 1974). An AGGA sequence is located 4 bp upstream of the ATG start codon (Fig. 3.2). A promoter-like sequence (Hawley and McClure, 1983) is located upstream of the ATG start codon (Fig. 3.2) but the putative -10 and -35 regions are separated by 22 bp (TTCTCT-N<sub>22</sub>-TAGAAT; Fig. 3.2). Two putative repressor binding sites homologous to the *gal* operator (O<sub>E</sub>; von Wilcken-Bergmann and Muller-Hill, 1982) are located in the intergenic region between the *scrA* and the *scrR* genes (Fig. 3.2).

**CODON USAGE:** The codon usage of the *scrR* gene was compared to that of 15 other *V. alginolyticus* genes (Table 3.1). The codon usage of the *scrR* gene did not reflect the bias of the other 15 *V. alginolyticus* genes. In particular the aa residues glycine, threonine, tyrosine and proline showed an absolute preference for GGC, ACA, UAU and CCG, respectively. The unusual codon usage of the *scrR* gene is consistent with the finding that it codes for a repressor since rare codon usage has been reported for a number of *E. coli* genes coding for regulatory proteins (Konigsberg and Godson, 1983).

**DEDUCED AA SEQUENCE AND HOMOLOGY COMPARISONS:** On the basis of identical aa sequence similarities, the *V. alginolyticus* Scr repressor protein showed 36, 31, and 28% similarity to the N-terminal regions of the *E. coli* Gal (von Wilcken-Bergmann and Muller-Hill, 1982), Lac (Farabaugh, 1978) and Cyt (Valentin-Hansen *et al.*, 1986) repressors, respectively. Interestingly, the Scr repressor (94 aa) was considerably smaller than the Gal (343 aa), Lac (360 aa) and Cyt (341 aa) repressors.

**TABLE 3.1 Codon usage<sup>a</sup> of the *scrR* gene and ORF5 compared to that of 15 *V. alginolyticus* genes (*Va*)<sup>b</sup>.**

Residue and codon	<i>scrR</i>	ORF5	<i>Va</i>	Residue and codon	<i>scrR</i>	ORF5	<i>Va</i>
Phe UUU	1	6	100	Tyr UAU	2	1	47
Phe UUC	0	1	100	Tyr UAC	0	2	91
Leu UUA	0	1	56	His CAU	1	6	38
Leu UUG	0	1	83	His CAC	0	6	51
Leu CUU	2	2	97	Gln CAA	4	4	145
Leu CUC	0	0	26	Gln CAG	2	4	76
Leu CUA	1	2	126	Asn AAU	0	1	49
Leu CUG	3	2	77	Asn AAC	3	1	127
Ile AUU	4	1	123	Lys AAA	9	1	154
Ile AUC	2	3	176	Lys AAG	2	2	79
Ile AUA	1	2	17	Asp GAU	4	2	133
Met AUG	1	1	149	Asp GAC	1	1	144
Val GUU	2	4	136	Glu GAA	5	2	176
Val GUC	3	2	47	Glu GAG	4	0	116
Val GUA	4	1	117	Cys UGU	1	0	30
Val GUG	4	0	82	Cys UGC	0	1	12
Ser UCU	1	1	95	Trp UGG	0	2	41
Ser UCC	0	0	13	Arg CGU	1	2	106
Ser UCA	3	0	57	Arg CGC	1	3	55
Ser UCG	0	1	46	Arg CGA	0	0	15
Ser AGU	1	2	45	Arg CGG	1	1	1
Ser AGU	1	0	57	Arg AGA	0	0	14
Pro CCU	0	1	59	Arg AGG	0	0	2
Pro CCC	0	1	9	Gly GGU	0	1	248
Pro CCA	0	1	98	Gly GGC	5	1	94
Pro CCG	1	2	32	Gly GGA	0	0	33
Thr ACU	0	2	68	Gly GGG	0	1	24
Thr ACC	0	1	42				
Thr ACA	3	2	72				
Thr ACG	0	2	59				
Ala GCU	2	4	130				
Ala GCC	2	2	46				
Ala GCA	3	1	166				
Ala GCG	3	2	137				

<sup>a</sup> The codon usage listed here represents the number of times a particular codon is used in the gene(s).

<sup>b</sup> The genes used to compile this list are the sucrose uptake-encoding gene (*scrA*), the sucrase-encoding gene (*scrB*; Scholle *et al.*, 1989), the fructokinase-encoding gene (*scrK*), the protease-encoding gene (*proA*; Deane *et al.*, 1989), the glutamine synthetase-encoding gene (*glnA*; Maharaj *et al.*, 1989), the NR<sub>II</sub>-encoding gene (*ntrB*; Maharaj *et al.*, 1989) and 9 genes (*uncA-uncI*) involved in the *unc* operon for ATP synthase (Krumholz *et al.*, 1989).



*E. coli* JA221(pVS116) *scrB* expression was inducible; sucrase activity was high in sucrose grown cultures and low in glucose grown cultures (Table 3.2). Deletion of ORF5 (pVS116), therefore, did not affect *scrB* expression suggesting that it was not essential for regulation of the *V. alginolyticus* sucrose utilization system. In *E. coli* JA221(pVS117), *scrB* expression was constitutive and sucrase activity was high in both sucrose and glucose grown cultures (Table 3.2). A deletion in the putative *scrR* gene (pVS117), therefore, destroyed regulation. This confirmed the sequence data which indicated that the *scrR* gene was located immediately upstream of the *scrA* gene and encoded an Scr repressor protein.

**TABLE 3.2** Sucrase specific activity for *E. coli* JA221 transformants after growth on MM supplemented with sucrose or glucose.

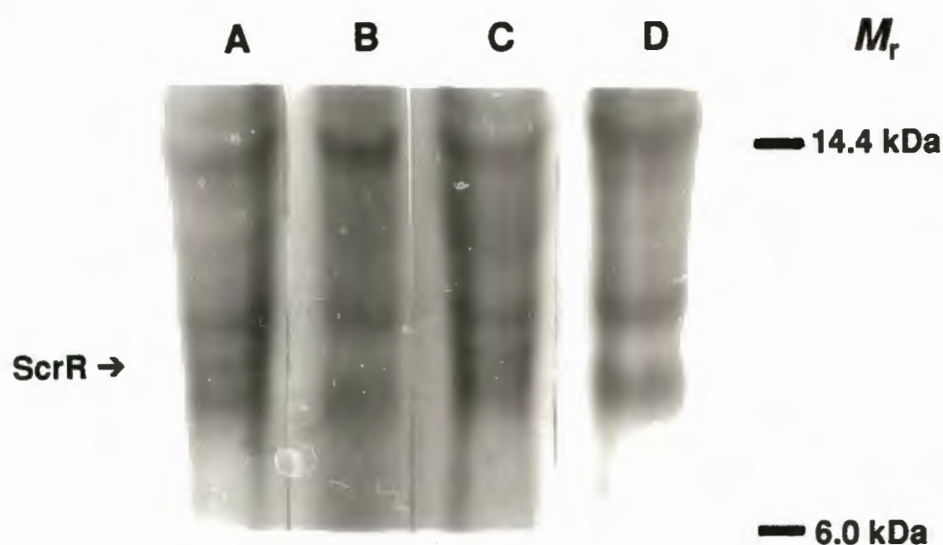
Strain	Sucrase specific activity <sup>a</sup>	
	sucrose	glucose
<i>E. coli</i> JA221(pVS100)	8.5	0.7
<i>E. coli</i> JA221(pVS103)	8.9	1.2
<i>E. coli</i> JA221(pVS116)	9.4	0.6
<i>E. coli</i> JA221(pVS117)	7.2	7.2
<i>E. coli</i> JA221(pVS118)	8.3	7.8
<i>E. coli</i> JA221(pVS118,SK)	8.1	7.3
<i>E. coli</i> JA221(pVS118,pVS119)	10.1	8.6
<i>E. coli</i> JA221(pVS118,pVS121)	9.6	8.9

<sup>a</sup> Sucrase specific activity was expressed as  $\mu$ moles reducing sugar per min per mg.

To further characterize the mechanism of regulation, *in trans* studies were conducted. Plasmid pVS118 contained the same insert fragment as pVS117 cloned into the vector pACYC177 (Fig. 3.1). In *E. coli* JA221(pVS118) and

*E. coli* JA221(pVS118,SK), *scrB* expression was constitutive as in *E. coli* JA221(pVS117) (Table 3.2). In *E. coli* JA221(pVS118,pVS119), *scrB* expression was also constitutive and sucrase activity was high in both sucrose and glucose grown cultures. Similar results were obtained for *E. coli* JA221(pVS118,pVS121) (Table 3.2). This result indicated that the *scrR* gene cloned on a high copy number plasmid (pVS119 or pVS121) was not able to regulate *in trans* the *V. alginolyticus* sucrose utilization system cloned on a lower copy number plasmid (pVS118). This suggested that the *scrR* deletion on plasmid pVS118 may have removed additional regulatory features required in the *cis* configuration in *E. coli* transformants.

**DNA-DIRECTED CELL-FREE PROTEIN SYNTHESIS:** To confirm that the *scrR* gene encoded a protein, the synthesis of proteins by plasmid pVS119 and deleted derivatives of pVS119 (pVS120 and pVS121; Fig. 3.1) was investigated in an *E. coli* DNA-directed cell-free system (Fig. 3.4). Cell-free coupled transcription-translation of pVS119 resulted in the production of a protein of apparent  $M_r$  of approximately 8 500, which was not produced by the vector Bluescript SK (Fig. 3.4). This protein was also produced by plasmid pVS121 (ORF5 deleted, *scrR* intact) but not by plasmid pVS120 (ORF5 intact, *scrR* deleted) indicating that it corresponded to the Scr repressor (Fig. 3.4). The apparent  $M_r$  of this protein approximated the predicted  $M_r$  for the *scrR* gene product (10 268).



**FIG. 3.4** Autoradiogram of the cell-free coupled transcription-translation polyacrylamide gel. Lanes A-D are the translation products of *in vitro* transcribed and translated plasmids: A, pVS121; B, pVS120; C, pVS119; D, Bluescript SK. The position of the Scr repressor is indicated (ScrR).

No protein corresponding to ORF5 could be detected; either no protein was coded for or if a protein was produced, vector-produced proteins of a similar  $M_r$  may have obscured its detection.

### 3.3.2 Nucleotide and deduced aa sequences of the *V. alginolyticus* ORF5

ORF5 also lies in the opposite orientation with respect to the *scrA* gene and is situated downstream of the *scrR* gene. This ORF consists of a presumptive ATG start codon and a TAA stop codon, and contains 297 bp which encode a polypeptide of 99 aa residues (Fig. 3.2). The  $M_r$  of the predicted polypeptide is 11 599. A classical ribosome-binding site (GAGGAG) is located 6 bp upstream of the ATG start codon (Fig. 3.2) (Shine and Dalgarno, 1974). A promoter-like sequence (Hawley and McClure, 1983) is located upstream of the ATG start codon (TTTACT-N<sub>14</sub>-TATTAT) (Fig. 3.2). The deduced aa sequence was found to contain a high proportion of histidine residues (12.1%). The codon usage, like that of the *scrR* gene, did not reflect the bias of the other *V. alginolyticus* genes (Table 3.1). The nt and deduced aa sequences were not significantly homologous with any of the sequences in the current databases accompanying the GCG package version 6.1. In section 3.3.1 it was shown that ORF5 was not involved in the regulation of the *V. alginolyticus* sucrose utilization system and was not essential for sucrose metabolism. The *S. typhimurium* (pUR400) sucrose utilization system contains an *scrY* gene which encodes a sucrose porin ( $M_r$  58 000). Insertional inactivation of *scrY* led to reduced, though not completely negative, sucrose fermentation (Schmid *et al.*, 1988). While the ScrY protein is considerably larger than the predicted protein for ORF5, it would nevertheless be interesting to compare their aa sequences when the deduced aa sequence of the *scrY* gene becomes available. It is also possible that ORF5 is not involved in sucrose metabolism but is part of another operon adjacent to the *V. alginolyticus* sucrose utilization system.

### 3.4 CONCLUSION

Nucleotide sequencing, deletion analysis and cell-free coupled transcription-translation located the *V. alginolyticus scrR* gene upstream of the *scrA* gene. This is the first report of the nt and deduced aa sequence of an Scr repressor-encoding gene from a Gram-negative bacterium. It will be very interesting to compare the *V. alginolyticus* Scr repressor with the regulatory proteins of the *S. typhimurium* and *S. mutans* sucrose utilization systems, when their sequences become available. Studies with the *V. alginolyticus* sucrose utilization system containing a deletion in the *scrR* gene indicated that the *scrR* deletion could not be complemented *in trans* in *E. coli* transformants. This suggested that the *scrR* gene, while encoding the Scr repressor, was also required *in cis* for the regulation of the *V. alginolyticus* sucrose utilization system in *E. coli*. Regulation of the *V. alginolyticus* sucrose utilization system may be a complex process involving distantly removed DNA structural features in addition to an Scr repressor. Complex regulatory mechanisms involving DNA looping, in addition to repressor/activator proteins, have been proposed for the galactose utilization operon (Majumdar and Adhya, 1984) and the arabinose utilization operon (Huo *et al.*, 1988), and appears to be a common regulatory strategy in prokaryotes (Eismann and Muller-Hill, 1990).

## CHAPTER FOUR

TRANSFORMATION OF THE *V. ALGINOLYTICUS* SUCROSE  
UTILIZATION SYSTEM INTO A *B. SUBTILIS sacA sacB* MUTANT

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## CHAPTER FOUR

TRANSFORMATION OF THE *V. ALGINOLYTICUS* SUCROSE UTILIZATION SYSTEM INTO A *B. SUBTILIS* *sacA sacB* MUTANT

**SUMMARY:** The sucrose utilization system of *V. alginolyticus* (*scrRAKB*) was subcloned into the *B. subtilis* shuttle vector pHV33 to generate pVS203, and transformed into *B. subtilis* QB602 (a *sacA sacB* mutant). The *V. alginolyticus* sucrose utilization system was not maintained in *B. subtilis* QB602 as the *V. alginolyticus* DNA was spontaneously deleted in *B. subtilis* QB602. Transformation of *B. subtilis* QB602 with pHV33 containing the *V. alginolyticus* *scrB* and *scrK* genes (pVS210) yielded 10-20% stable transformants which produced high levels of sucrose activity. These stable transformants may be the result of a putative recombination event between the *scrB* gene and the chromosomal *sacA* gene. The remaining 80-90% of the transformants produced low levels of sucrose activity. The low levels of sucrose production represented the actual level of expression of the *V. alginolyticus* *scrB* gene in *B. subtilis* QB602(pVS210).

## 4.1 INTRODUCTION

Although *V. alginolyticus* is classified as a Gram-negative bacterium it has been found to be similar to the Gram-positive *Bacillus* in a number of aspects. These aspects have been dealt with in the General Introduction to *V. alginolyticus* (section 1.1) and have been summarized here. Both *V. alginolyticus* and *Bacillus* strains have been found to produce true extracellular proteases during the stationary growth phase; protease production was rifampin-insensitive and was subject to end-product and catabolite repression which was not relieved by cAMP (Reid *et al.*, 1978; Reid *et al.*, 1980; Long *et al.*, 1981). The aa sequence of a calcium-dependent, SDS-resistant alkaline serine exoprotease (protease A) from *V. alginolyticus* showed significant homology to *Bacillus* alkaline serine exoproteases, but low homology to the alkaline serine exoprotease produced by a more closely related Gram-negative *S. marcescens* strain (Deane *et al.*, 1989). In *B. subtilis* and *V. alginolyticus*, histidine was found to be the inducer of the histidine utilization (Hut) pathway (Chasin and Magasanik, 1968; Bowden *et al.*, 1982), whereas in the Gram-negative enteric bacteria, urocanic acid was found to be the inducer of the Hut enzymes (Smith and Magasanik, 1971). An investigation of the nitrogen catabolic enzymes of *V. alginolyticus* revealed that they were not regulated by nitrogen catabolite repression (Bodasing *et al.*, 1983). In this respect *V. alginolyticus* again resembled *Bacillus* strains rather than the more closely related Gram-negative enteric bacteria.

The identical aa similarity between the *V. alginolyticus* and the *B. subtilis* sucrase enzymes (34%) was significantly higher than the identical aa similarity observed when the sucrases from the two Gram-positive bacteria *B. subtilis* and *S. mutans* were compared to each other (17%) (Fouet *et al.*, 1986; Sato and Kuramitsu, 1988; Scholle *et al.*, 1989). At the DNA level there was 47% similarity between the *B. subtilis* and *V. alginolyticus* sucrase-encoding structural genes. In view of these physiological and sequence similarities between *V. alginolyticus* and *B. subtilis*, expression of the *V. alginolyticus* sucrose utilization system in *B. subtilis* was investigated. To this end, the *V. alginolyticus* sucrose utilization system and the *scrB* gene were transformed into a *B. subtilis* sucrase (*sacA*) and levansucrase (*sacB*) mutant.

## 4.2 MATERIALS AND METHODS

### **4.2.1 Bacterial strains and plasmids**

*E. coli* JA221 (Table 2.1) and *B. subtilis* QB602 *sacA321 sacB209 hisA1* (a gift from A. Fouet, Department of Biotechnology, Institut Pasteur, France) were used to study sucrose production. Plasmids pVS103 and pVS110 have been described in section 2.2.3 and 2.2.1, respectively. The *V. alginolyticus* sucrose utilization system was subcloned into the *B. subtilis* shuttle vector pHV33 (Primrose and Ehrlich, 1981) (Appendix B). Plasmid pBSG8-1 (a gift from A. Fouet, Department of Biotechnology, Institut Pasteur, France) contained the *B. subtilis sacA* gene and a truncated *sacP* gene cloned into pHV33 (Fouet *et al.*, 1982). Plasmids pVS203 and pVS210 were constructed in this study.

### **4.2.2 Standard methods, media, buffers and solutions**

Standard methods, media, buffers and solutions are described in Appendix A.

### **4.2.3 Subcloning the sucrose utilization system**

Plasmid pVS103 containing the *V. alginolyticus* sucrose utilization system was subcloned into pHV33; the 2.9-kb *HindIII-HindIII* restriction endonuclease fragment of pHV33 was gel purified (Appendix A) and ligated to *HindIII* restriction endonuclease digested pVS103 (9.5 kb) to generate pVS203 (Fig. 4.1). Plasmid pVS110 contained the 3.8-kb *PstI-HpaI* restriction endonuclease fragment of pVS103 (containing the *V. alginolyticus scrB* and *scrK* genes) subcloned into the multiple cloning cassette of Bluescript SK. This restriction endonuclease fragment was subcloned using the *SalI* restriction endonuclease site in the multiple cloning cassette of pVS110. The 3.8-kb *SalI*(cloning cassette)-*PstI* restriction endonuclease fragment of pVS110 was ligated to the 4.6-kb *SalI-PstI* restriction endonuclease fragment of pHV33 to generate pVS210 (Fig. 4.1). This subcloning procedure removed the *E. coli* origin of replication of pHV33 so that successful ligation products containing the *V. alginolyticus scrB* and *scrK* genes subcloned into pHV33

could only be selected for in *B. subtilis*. *E. coli* and *B. subtilis* were transformed as described in Appendix A. *E. coli* JA221 transformants were selected on LB or sucrose MacConkey agar containing chloramphenicol (Cm) (10 µg/ml) (Appendix A). *B. subtilis* QB602 transformants were initially selected on DM3 plates containing Cm (10 µg/ml) (Appendix A). Sucrase-positive *B. subtilis* QB602 transformants were selected by replica plating onto C mineral medium (CMM) agar plates supplemented with sucrose and Cm (5 µg/ml) (Appendix A). Plasmid DNA was isolated from *B. subtilis* QB602 transformants by a modification of the procedures described for *E. coli* transformants (Appendix A). All other experimental details necessary for subcloning have been mentioned in section 2.2.3.

#### 4.2.4 Nucleotide sequencing

DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method (Appendix A). A 15-mer primer (5'-ATATCTTTGCTCTAC-3') which corresponded to positions +25-+39 of the *scrB* gene was used.

#### 4.2.5 Sucrase assays

Sucrase assays were carried out on late-exponential-phase cultures of *E. coli* JA221 and *B. subtilis* QB602 transformants ( $A_{600}=1.0$ ). Samples were assayed in duplicate and experiments were repeated three times.

Cells (20 ml) were grown at 37°C in MM (*E. coli* JA221 transformants) or CMM (*B. subtilis* QB602 transformants) supplemented with sucrose and Cm (10 µg/ml for *E. coli* JA221 transformants; 5 µg/ml for *B. subtilis* QB602 transformants) (Appendix A). For cured *B. subtilis* QB602 cells, Cm was omitted. Cell extracts were prepared from these cells as described in section 2.2.7. Sucrase assays were performed on the cell extracts by a modification of the method described by Scholle *et al.* (1987) as outlined in section 2.2.7. Sucrase specific activity was expressed as µmoles of reducing sugar produced per min per mg protein.

#### 4.2.6 Protein assays

Protein concentrations were determined by the method of Bradford (1976) as described in section 2.2.8.

#### 4.2.7 Plasmid curing

*B. subtilis* QB602 transformants were cured by overnight growth without antibiotic selection in brain heart infusion broth (Merck). Cured cells were selected by replica plating on brain heart infusion agar plates with and without Cm (10 µg/ml). Brain heart infusion agar plates were prepared using brain infusion broth (Merck) and agar (1.5%, w/v). Cured cells selected on the basis of their Cm sensitivity (Cm<sup>S</sup>) by replica plating, were checked for the presence of plasmids by the small scale plasmid isolation method (Appendix A).

#### 4.2.8 Southern hybridization studies

Plasmids pBSG8-1 and pVS210, and *B. subtilis* chromosomal DNA, prepared as described in Appendix A, were digested with restriction endonucleases and the DNA fragments separated by electrophoresis in TBE buffered 0.8% agarose gels (Appendix A). The DNA was transferred to Hybond N+ membranes (Amersham) by the DNA/RNA alkali blotting procedure (Appendix A). The nonradioactive DNA labelling and detection kit from Boehringer Mannheim (catalogue number 1093657) was used for the preparation of digoxigenin-dUTP labelled probes and their hybridization to the immobilized DNA on the membrane (Appendix A). The manufacturer's procedures for DNA labelling, hybridization and probe detection were followed precisely. The procedure for hybridization involved high stringency washes of the hybridized DNA. The membranes were washed twice for 5 min at room temperature with 2 x SSC (Appendix A), 0.1% SDS (w/v) and twice for 15 min at 68°C with 0.1 x SSC, 0.1% SDS (w/v).

### 4.3 RESULTS AND DISCUSSION

#### 4.3.1 Subcloning and deletion of the *V. alginolyticus* sucrose utilization system in *B. subtilis*

The *V. alginolyticus* sucrose utilization system on plasmid pVS203 (Fig. 4.1) was transformed into *E. coli* JA221. The *E. coli* JA221(pVS203) cells expressed the *V. alginolyticus* sucrose utilization system and were able to utilize sucrose as the sole source of carbon. Plasmid pVS203 was transformed into *B. subtilis* QB602 and 50 Cm resistant (Cm<sup>R</sup>) transformants selected on DM3 agar plates were replica plated onto sucrose CMM agar plates. None of the Cm<sup>R</sup> resistant colonies were able to grow on the sucrose CMM agar plates. Extraction and analysis of pVS203 from *B. subtilis* QB602(pVS203) indicated that the pVS203 recombinant plasmid was unstable in *B. subtilis* QB602(pVS203) and the 9.5-kb DNA fragment containing the sucrose utilization system was spontaneously deleted leaving a 2.9-kb plasmid. This plasmid probably corresponded to the 2.9-kb *Hind*III-*Hind*III restriction endonuclease fragment of pHV33.

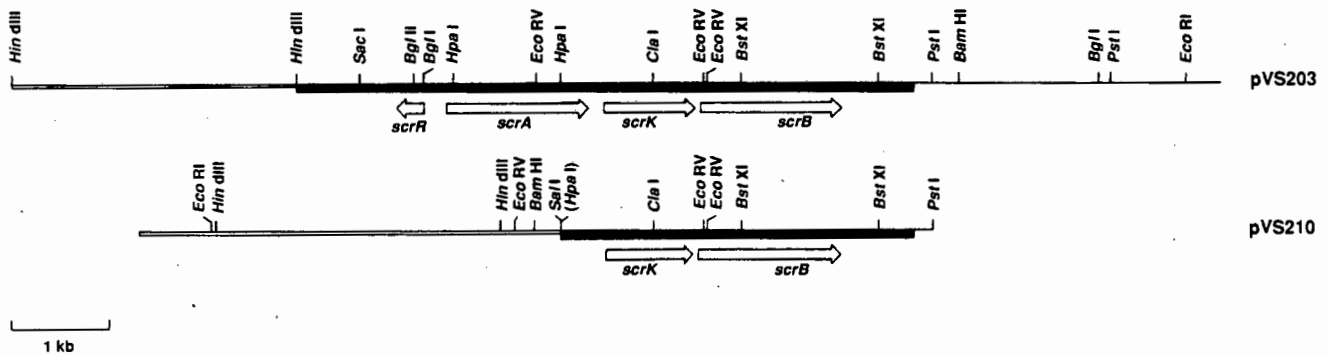


FIG. 4.1 Restriction maps of pVS203 and pVS210. The open, bold and thin lines represent pHV33 vector DNA, *V. alginolyticus* DNA and pEcoR251 vector DNA, respectively. The open arrows represent the ORF's.

#### 4.3.2 Subcloning, expression and putative recombination of the *V. alginolyticus* *scrB* gene in *B. subtilis*

**SUBCLONING AND EXPRESSION:** The *Sall*-*Pst*I restriction endonuclease digestion products of pVS110 were ligated to the *Sall*-*Pst*I restriction endonuclease digestion products of pHV33 and the ligation mixture transformed into *B. subtilis* QB602. Cm<sup>R</sup> *B. subtilis* QB602 colonies were selected on DM3 agar plates and replica plated onto sucrose CMM agar plates to screen for sucrase-positive clones. Fifty Cm<sup>R</sup> colonies were screened for growth on the sucrose plates and only one was able to grow. The plasmid DNA was extracted and found to contain the 3.8-kb *Sall*-*Pst*I restriction endonuclease fragment of pVS110 (containing the *V. alginolyticus* *scrB* and *scrK* genes) inserted into the 4.6-kb *Sall*-*Pst*I restriction endonuclease fragment of pHV33 (pVS210; Fig. 4.1). Retransformation of pVS210 into *B. subtilis* QB602 resulted in 10-20% of the retransformants being sucrase-positive, Cm<sup>R</sup> and able to grow on sucrose CMM agar plates. Since the retransformation was carried out with purified plasmid pVS210, the proportion of sucrase-positive transformants obtained (10-20%) was greater than the number of sucrase-positive transformants initially obtained with the ligation mixture (2%). The appearance of sucrase-positive colonies was not due to back mutations on the chromosome of *B. subtilis* QB602 at the *sacA321 sacB209* loci, since control transformation experiments with pHV33 never yielded sucrase-positive colonies. The plasmid DNA from the 80-90% apparently sucrase-negative *B. subtilis* QB602(pVS210) Cm<sup>R</sup> retransformants which were unable to grow on sucrose CMM agar plates, was extracted and shown to have the same restriction endonuclease sites as pVS210. The production of sucrase by the stable sucrase-positive *B. subtilis* QB602(pVS210) transformants and the apparently sucrase-negative *B. subtilis* QB602(pVS210) transformants was determined in liquid sucrose CMM (supplemented with 0.25% casamino acids, w/v, to aid growth of the apparently sucrase-negative transformants; Appendix A). The stable sucrase-positive *B. subtilis* QB602(pVS210) cells produced high levels of sucrase activity while the apparently sucrase-negative *B. subtilis* QB602(pVS210) cells produced low levels of sucrase activity (Table 4.1). Sucrase activity was never detected in control *B. subtilis* QB602(pHV33) cells (Table 4.1).

The apparently sucrase-negative *B. subtilis* QB602(pVS210) cells obtained after transformation and producing low levels of sucrase activity appeared to be relatively stable. Sucrase-positive *B. subtilis* QB602(pVS210) cells producing high levels of sucrase activity were never obtained after growth of the apparently sucrase-negative *B. subtilis* QB602(pVS210) cells in liquid sucrose CMM supplemented with casamino acids.

**NUCLEOTIDE SEQUENCING:** To determine whether the high level of sucrase production in the stable sucrase-positive transformants involved a change in the DNA upstream of the start of the *V. alginolyticus scrB* gene which caused over expression of the *scrB* gene, the nt sequences of this region on plasmids pVS110 and pVS210 were determined. The nt sequences of the *scrB* upstream regions of pVS110 and pVS210 were identical to one another and were also identical to the sequence of the *V. alginolyticus scrB* upstream region reported by Scholle *et al.* (1989) (see also Fig. 2.2 in section 2.3.1).

**CURING EXPERIMENTS:** Since the *sacA* and the *scrB* genes showed 47% nt similarity, a recombination event may have occurred to generate the stable sucrase-positive *B. subtilis* QB602(pVS210) transformants. To determine whether the stable sucrase-positive *B. subtilis* QB602(pVS210) Cm<sup>R</sup> cells resulted from recombination, the cells were cured of their plasmids. Cured *B. subtilis* QB602 cells which were Cm<sup>S</sup> and did not contain detectable plasmids, showed similar levels of sucrase activity as the Cm<sup>R</sup> cells containing plasmids (Table 4.1). This suggested that a putative recombination event may have occurred in the sucrase-positive transformants that either repaired the chromosomal *B. subtilis* QB602 *sacA* gene, or resulted in incorporation into the *B. subtilis* QB602 genome of one of the plasmid copies of the entire *V. alginolyticus scrB* gene. Therefore, the high sucrase activity in these sucrase-positive transformants may either be due to expression of the repaired *sacA* gene or expression of the chromosomally incorporated *scrB* gene. Control experiments involving the curing of *B. subtilis* QB602(pHV33) cells of pHV33 never yielded sucrase positive colonies.

**TABLE 4.1 Sucrase activity of sonicated cell extracts obtained from cured and uncured *B. subtilis* QB602 transformants after growth in liquid sucrose CMM supplemented with casamino acids.**

Strain	Sucrase specific activity <sup>a</sup>	
	Uncured	Cured
QB602(pHV33)	0.00	0.00
QB602(pVS210) <sup>b</sup>	7.40	5.90
QB602(pVS210) <sup>c</sup>	0.35	0.00

<sup>a</sup> Sucrase specific activity was expressed as  $\mu$ mole reducing sugar per min per mg.

<sup>b</sup> Corresponds to the stable sucrase-positive transformants.

<sup>c</sup> Corresponds to the apparently sucrase-negative transformants.

Similar curing experiments were carried out on the apparently sucrase-negative *B. subtilis* QB602(pVS210) Cm<sup>R</sup> cells. Cured *B. subtilis* QB602 cells which were Cm<sup>S</sup> and did not contain detectable plasmids, did not produce detectable sucrase activity (Table 4.1). This indicated that the low sucrase activity of the apparently sucrase-negative transformants was due to the expression of the *V. alginolyticus* *scrB* gene on pVS210.

**SOUTHERN HYBRIDIZATION STUDIES:** The sucrase-positive *B. subtilis* QB602(pVS210) cells cured of pVS210 still produced high levels of sucrase, and pVS210 extracted from stable sucrase-positive *B. subtilis* QB602(pVS210) cells retransformed 10-20% of *B. subtilis* QB602 cells to be sucrase-positive. This suggested that a putative recombination event may have occurred that either repaired the *sacA* mutation on the chromosome of *B. subtilis* QB602 cells (but did not impair the sucrase phenotype of the plasmid *scrB* gene), or resulted in incorporation of one of the plasmid copies of the *V. alginolyticus* *scrB* gene into the *B. subtilis* QB602 genome. Southern hybridization studies were carried out to determine whether this putative recombination event involved large DNA fragments or insertion of the entire *scrB* gene. Sucrase-positive *B. subtilis* QB602(pVS210),

apparently sucrase-negative *B. subtilis* QB602(pVS210) and *B. subtilis* QB602(pHV33) were cured of their plasmids and chromosomal DNA prepared from the resulting *B. subtilis* QB602 cells. To investigate whether the putative recombination event involved large DNA fragments of the chromosomal *sacA* gene, the 3 chromosomal DNA's and plasmid pBSG8-1 were each digested separately with *Ava*I and *Bgl*II. To investigate whether the putative recombination event involved large DNA fragments of the *V. alginolyticus* *scrB* gene or the entire *scrB* gene, all 3 chromosomal DNA's and plasmid pVS210 were double digested with *Bst*XI and *Eco*RV. Two separate hybridization experiments were carried out using nonradioactively labelled pBSG8-1 as a probe in the first case and nonradioactively labelled pVS210 as a probe in the second case. To ensure that hybridization only occurred between the nonradioactively labelled probes and homologous DNA fragments immobilized on the membrane, high stringency washes were used.

The *Ava*I and *Bgl*II restriction endonuclease sites of the *sacA* gene were chosen since they were located in regions of the *B. subtilis* *sacA* gene that were homologous to the *V. alginolyticus* *scrB* gene and therefore were possible recombination sites. The *B. subtilis* *sacA* gene contains a single internal *Ava*I restriction endonuclease fragment (1060 bp) and 2 internal *Bgl*II restriction endonuclease fragments (600 bp and 230 bp) (deduced from published gene sequence [Fouet *et al.*, 1986]). *Ava*I and *Bgl*II restriction endonuclease digested pBSG8-1 produced the desired *sacA* internal restriction endonuclease fragments which were detected when probed with nonradioactively labelled pBSG8-1 (Fig. 4.2A; lanes 1 and 5). *Ava*I and *Bgl*II restriction endonuclease digested chromosomal DNA from all 3 cured strains were found to contain the identical *sacA* internal restriction endonuclease fragments when probed with nonradioactively labelled pBSG8-1 (Fig. 4.2A; lanes 2-4 and 6-8). The above results therefore indicated that the putative recombination event that occurred on the chromosome of sucrase-positive *B. subtilis* QB602(pVS210) did not cause major rearrangements of the *sacA* gene and therefore did not involve large DNA fragments.

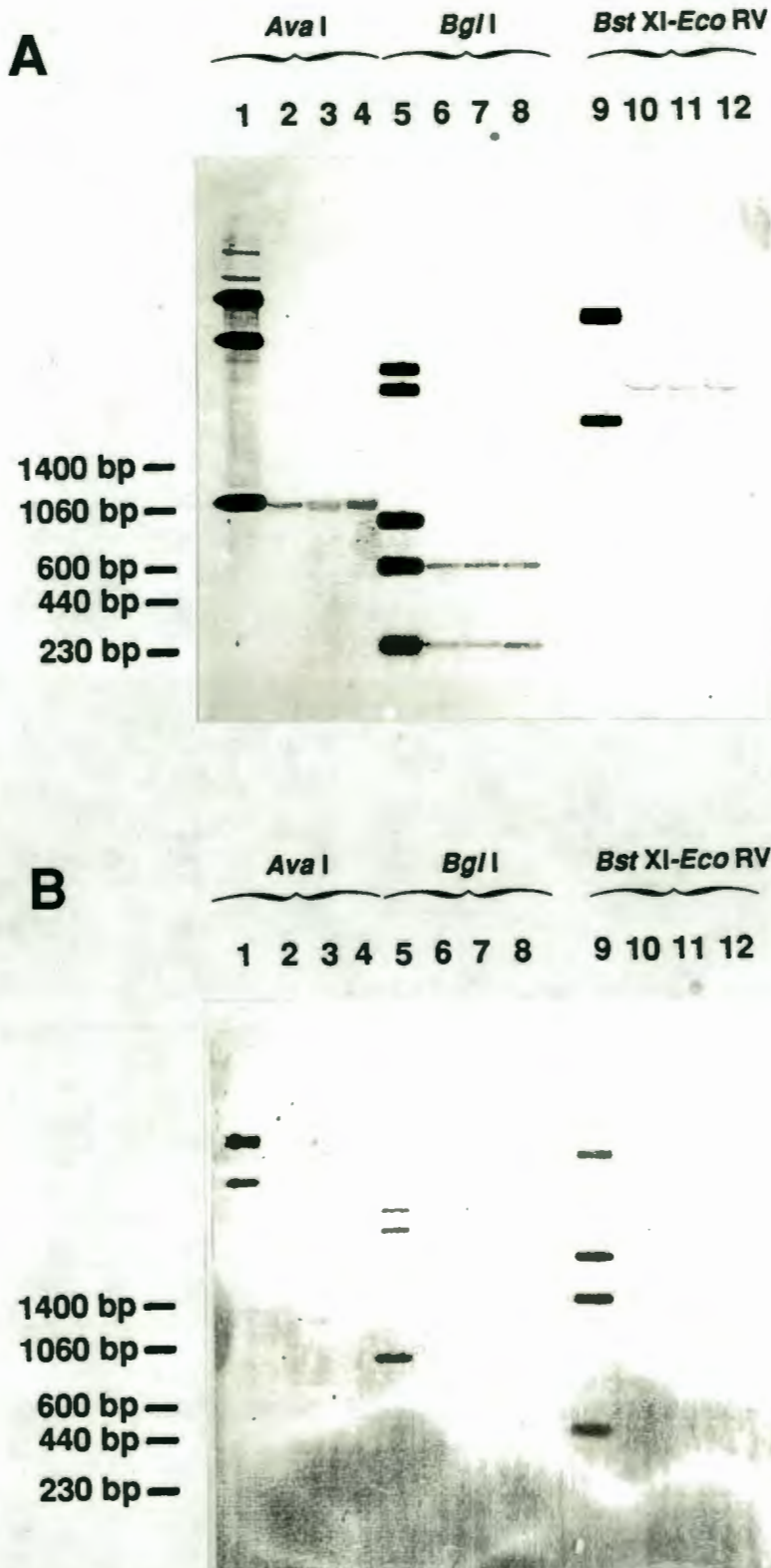


FIG. 4.2 Hybridization of nonradioactively labelled pBSG8-1 (A) and nonradioactively labelled pVS210 (B) with membrane immobilized pBSG8-1, pVS210 and chromosomal DNA obtained from cured *B. subtilis* QB602 transformants. Lanes 1 and 5, *Ava*I and *Bgl*II restriction endonuclease digested pBSG8-1, respectively; lane 9, *Bst*XI-*Eco*RV restriction endonuclease digested pVS210; lanes 2-4, 6-8, 10-12, *Ava*I, *Bgl*II, *Bst*XI-*Eco*RV restriction endonuclease digested chromosomal DNA, respectively. Chromosomal DNA was obtained from cured sucrose-positive *B. subtilis* QB602(pVS210) (lanes 2,6,10), cured apparently sucrose-negative *B. subtilis* QB602(pVS210) (lanes 3,7,11) and cured *B. subtilis* QB602(pHV33) (lanes 4,8,12).

The *V. alginolyticus scrB* gene contains 2 internal *BstXI-EcoRV* restriction endonuclease fragments of 440 bp and 30 bp (Figs 2.1 and 4.1), however, the 30-bp restriction endonuclease fragment was too small to be detected after gel electrophoresis. *BstXI-EcoRV* restriction endonuclease digested pVS210 produced the desired 440-bp restriction endonuclease fragment which was detected when probed with nonradioactively labelled pVS210 (Fig. 4.2B; lane 9). *AvaI*, *BglI* and *BstXI-EcoRV* restriction endonuclease digested chromosomal DNA from all 3 cured strains did not produce any bands when probed with nonradioactively labelled pVS210 (Fig. 4.2B; lanes 2-4, 6-8 and 10-12). Therefore large DNA fragments of the *scrB* gene or the entire *scrB* gene had not recombined onto the chromosome of the sucrose-positive *B. subtilis* QB602(pVS210). This was consistent with the previous result which suggested that the putative recombination event did not involve large DNA fragments

Although the *V. alginolyticus scrB* gene and the *B. subtilis sacA* gene share 47% DNA similarity, the high stringency washes used in these hybridization experiments avoided cross-hybridization. Hence, when pBSG8-1 was used as a probe, hybridization only occurred between pBSG8-1 and the membrane immobilized vector containing DNA fragments of pVS210 cut with *BstXI-EcoRV* (Fig. 4.2A; lane 9). Similarly, when pVS210 was used as a probe, hybridization only occurred at the membrane immobilized vector containing DNA fragments of pBSG8-1 cut with *AvaI* or *BglI* (Fig. 4.2B; lanes 1 and 5).

#### 4.4 CONCLUSION

The 9.5-kb DNA fragment of pVS203, containing the *V. alginolyticus* sucrose utilization system, was spontaneously deleted when pVS203 was transformed into *B. subtilis* QB602. Instability of heterologous DNA cloned into *B. subtilis* shuttle vectors is a well recognized phenomenon (Peeters *et al.*, 1988) and the results obtained here with pVS203 is another example of this instability. The instability appeared to be related to the size of the cloned *V. alginolyticus* DNA since the smaller DNA fragment (3.8 kb) containing the *scrB* and *scrK* genes (plasmid pVS210) was not deleted in *B. subtilis* QB602(pVS210).

The high sucrase activity of the stable sucrase-positive *B. subtilis* QB602(pVS210) transformants may be due to a putative recombination event between the *scrB* gene and the chromosomal *sacA* gene. The putative recombination event did not involve the incorporation of the entire *V. alginolyticus scrB* gene (or large fragments of the *scrB* gene) into the *B. subtilis* QB602 chromosome. The putative recombination event may have repaired the chromosomal *sacA* mutation, however, the putative recombination event did not involve large DNA fragments. Therefore, the high sucrase activity observed for these transformants is likely to represent expression of the repaired chromosomal *sacA* gene of *B. subtilis* QB602 and not expression of the *V. alginolyticus scrB* gene on pVS210. It must be stressed that this possible explanation of a complex phenomenon is very preliminary and would require further experimental evidence, including sequencing the putative repaired *sacA* gene. Since the observed phenomenon is of uncertain importance and as there are other more important areas of research requiring investigation it is unlikely that time and resources will be allocated to a complete characterization of this phenomenon.

The putative recombination event did not occur in the apparently sucrase-negative *B. subtilis* QB602(pVS210) transformants which produced low levels of sucrase activity. Therefore, the low levels of sucrase activity in these transformants represented inefficient expression of the *V. alginolyticus scrB* gene on pVS210. The promoter region located upstream of the *scrK* gene (section 2.3.2) may be used

poorly by the *B. subtilis* RNA polymerase, resulting in the low levels of sucrase activity.

It appears that for some unknown reason the putative recombination event only occurred at a frequency of 10-20% following transformation. The putative recombination event did not occur in the apparently sucrase-negative *B. subtilis* QB602(pVS210) cells which were stable and did not give rise to sucrase-positive cells producing high levels of sucrase activity.

## CHAPTER FIVE

### GENERAL CONCLUSION

Prior to this research, the *V. alginolyticus* sucrose utilization system was cloned into *E. coli* and shown to consist of a sucrase-encoding gene (*scrB*) and a sucrose uptake-encoding region (Scholle *et al.*, 1987; 1989). The sucrose utilization system was shown to be regulated and the nt sequence of the *scrB* gene determined. In addition, a wealth of research has shown that *V. alginolyticus* resembles *Bacillus* strains in a number of aspects. This study was aimed in general at extending the *V. alginolyticus* knowledge base with a specific focus on the sucrose utilization system. To this end the complete nt sequence of the *V. alginolyticus* sucrose utilization system was determined and the regions encoding the sucrose transport and regulatory functions were dissected at the molecular level. In view of the similarities between *V. alginolyticus* and *Bacillus* strains the expression of the *V. alginolyticus* sucrose utilization system in *B. subtilis* was investigated.

The results of this study have shown that the *V. alginolyticus* sucrose utilization system involves, in addition to a sucrase (gene *scrB*), a sucrose transport protein (EII<sup>Scr</sup>) of the PEP-dependent PTS (gene *scrA*), a fructokinase (gene *scrK*) and a repressor (gene *scrR*). The *V. alginolyticus* sucrose utilization system appears to resemble the pUR400-encoded system of *S. typhimurium* which also involves a sucrase, an EII<sup>Scr</sup> protein, a fructokinase and a repressor. However, the sucrose utilization system of *S. typhimurium* (pUR400 system) also involves a sucrose porin (gene *scrY*). By analogy to the pUR400-encoded sucrose utilization system, *V. alginolyticus* most likely transports sucrose into the cell as sucrose-phosphate *via* the EII<sup>Scr</sup> protein of the PTS. The intracellular sucrose-phosphate would be cleaved by the sucrase to generate glucose-phosphate and fructose. The fructokinase would

act on the fructose to produce fructose-phosphate. Both glucose-phosphate and fructose-phosphate would then be available to enter the carbohydrate metabolic pathways. Sucrose metabolism in *V. alginolyticus* is most likely negatively controlled by the repressor, however, the mechanism of regulation is unclear and awaits further investigation. Such an investigation would involve a detailed analysis of the promoter/operator in the intergenic region between the *scrA* and *scrR* genes. The binding conditions for the repressor need to be established by gel retardation studies thereby enabling the exact location of the operator site(s) to be determined by DNA footprinting. The regulatory mechanism appears to be a complex process involving negative control by the repressor while also being sensitive to positive control by CAP. In this regard, it was unexpected that no strong CAP binding site was located in the promoter/operator region.

Only a preliminary investigation of the expression of the *V. alginolyticus* sucrose utilization system in *B. subtilis* was possible. An investigation of the expression of the entire sucrose utilization system was hindered due to instability of the cloned DNA in *B. subtilis*. The *scrB* gene cloned on a smaller DNA fragment was found to be poorly expressed in *B. subtilis*. These results have indicated that while *V. alginolyticus* and *Bacillus* strains share similar characteristics, there may well be a number of differences at the level of transcription and translation. Consistent with this was the finding that the overall codon usage for 15 *V. alginolyticus* genes was more similar to that of *S. typhimurium* and *E. coli* genes than that of *B. subtilis* genes. It is most likely that *V. alginolyticus* promoters are poorly recognized in *B. subtilis*. This could be investigated by cloning the *V. alginolyticus scrB* gene downstream of various *V. alginolyticus* promoters. A number of *V. alginolyticus* genes and their promoters have been cloned. Expression of the promoters could be investigated in a *B. subtilis sacA sacB* mutant. Sucrase activity is easily assayed and could provide a simple measure of promoter strength.

## APPENDIX A

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## APPENDIX A

### STANDARD METHODS, MEDIA, BUFFERS AND SOLUTIONS

#### A.1 STANDARD METHODS

##### **A.1.1 Small scale isolation of plasmid DNA (miniprep)**

Plasmid was isolated from a 5 ml overnight culture (LB + Ap, 100 µg/ml) as described by Ish-Horowitz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 200 µl Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 400 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The sample was vortexed briefly and placed on ice for 5 min, before the addition of 300 µl ice-cold Solution III (5 M KOAc, pH 4.8). The sample was vortexed briefly, and, after 5 min on ice, cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (750 µl) was removed to a fresh tube, an equal volume of isopropanol was added and the DNA precipitated by centrifugation for 5 min. The DNA pellet was resuspended in TE (600 µl) (Appendix A.2.3) before adding NaClO<sub>4</sub> (60 µl, 5 M) and an equal volume of isopropanol, and precipitating the DNA by centrifugation for 15 min. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 50 µl TE buffer.

This procedure was used for both *E. coli* and *B. subtilis*, however, in the case of *B. subtilis* lysozyme (5 mg/ml) was added to Solution I.

### A.1.2 Large scale isolation of plasmid DNA (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 6 000 x g for 5 min and then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 12 000 x g for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 27 000 x g for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopycnic CsCl-EtBr ultracentrifugation (Maniatis *et al.*, 1982). The plasmid preparation was prepared for ultracentrifugation by the addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 27 000 x g for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.2 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated from the CsCl solution by the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10 µl (diluted in TE) between 220 and 310 nm. The concentration was determined by using the relationship  $A_{260} = 1$  for 50 µg/ml double-stranded DNA.

This procedure was used for both *E. coli* and *B. subtilis*, however, in the case of *B. subtilis* lysozyme (5 mg/ml) was added to Solution I.

### A.1.3 Extraction of chromosomal DNA from *B. subtilis*

A modification of the method of Marmur (1961) was used. A 200 ml culture was grown overnight in brain heart infusion broth (Merck) at 37°C in the presence of a suitable antibiotic. The cells were harvested by centrifugation at 6000 x g, washed in 1 x SSC and resuspended in 50 ml 1 x SSC. Lysozyme (2.5 ml, 0.5 mg/ml) was added to the cell suspension and the mixture was incubated for 1 h at 37°C to allow lysis to occur. Pronase (0.1 ml, 10 mg/ml) was added to the lysate and the mixture was poured into a dialysis bag and dialysed overnight against 1 x SSC. After dialysis the lysate was gently mixed with an equal volume (approximately 50 ml) of chloroform-isoamyl alcohol (24:1, v/v) chilled to 4°C. The milky solution was centrifuged at 2000 x g for 10 min causing separation into two layers. The bottom layer was removed with a Pasteur pipette and the chloroform-isoamyl treatment repeated on the aqueous layer until it was clear and there was no protein at the interface. The nucleic acids were precipitated by gently layering 2 volumes of chilled 95% ethanol on the aqueous phase. These layers were gently mixed with a glass rod causing the nucleic acids to "spool" onto the rod. The precipitate was allowed to disperse overnight in 0.1 x SSC (10 ml). RNA was removed by incubation for 2 h at 37°C with RNase (1 ml, 0.5 mg/ml). The RNase was first incubated at 80°C for 10 min to destroy any DNase activity which might have been present in the enzyme preparation. The DNA was precipitated by the addition of 1 volume of chilled isopropanol and the precipitate collected by spooling and centrifugation, and dissolved in a minimum volume of TE buffer. The DNA was stored under chloroform at 4°C.

### A.1.4 Exonuclease III digestion

Exonuclease III digestion was carried out by a modification of the method of Henikoff (1984). Ten sample tubes containing S1 mixture (25 µl; Appendix A.2.3) were prepared immediately before starting the exonuclease III digestion, and were kept on ice. Plasmid DNA (10 µg) previously double digested with appropriate restriction endonucleases and resuspended in 100 µl exobuffer (Appendix A.2.3), was equilibrated at 37°C for 5 min. A sample (9 µl) was first removed to a tube

containing S1 mixture before any exonuclease III was added. Exonuclease III (500 units, Boehringer Mannheim) was added to the equilibrated DNA, the mixture was briefly vortexed and after a lag period of 30 s 9 samples (9  $\mu$ l) were removed at regular time intervals. Each sample was transferred immediately to a tube containing S1 mixture. Once all the samples had been taken, the tubes were incubated at room temperature for 30 min, before the S1 nuclease reaction was terminated by the addition of S1 stop solution (3.4  $\mu$ l; Appendix A.2.3) to each tube. The tubes were then incubated at 70°C for 10 min. The exonuclease III-generated ends were filled in by the addition of 1 unit per tube of Klenow enzyme (Boehringer Mannheim) in Klenow buffer (3.4  $\mu$ l; Appendix A.2.3), incubation at room temperature for 3 min, followed by a further incubation for 5 min in the presence of a mixture of each dNTP (0.125 mM each). A sample of the shortened DNA (10  $\mu$ l) from each tube was blunt end ligated (Appendix A.1.7) in a large volume (50  $\mu$ l final volume). The deletions were transformed into *E. coli* LK111 (Appendix A.1.9) and the transformants were selected on LB agar (Appendix A.2.1) containing Ap (100  $\mu$ g/ml).

#### A.1.5 Restriction endonuclease digestion

Restriction endonuclease digestion was carried out as described by Maniatis *et al.* (1982). Restriction endonuclease digestions were performed in a total volume of 20  $\mu$ l in the presence of 3 units of restriction endonuclease enzyme per  $\mu$ g of DNA. Restriction endonuclease buffers obtained from the suppliers of the restriction endonucleases (Anglian, Boehringer Mannheim and Amersham), were used as recommended. Restriction endonuclease digestions were incubated at the recommended temperatures for 1-5 h.

For electrophoretic analysis, the digestions were terminated by the addition of 5  $\mu$ l DNA loading solution (Appendix A.2.3) to the 20  $\mu$ l digestions. If the digestion products were to be ligated, or filled in before ligation, they were purified by a phenol-ether extraction. The digestion products were diluted with sterile distilled water (380  $\mu$ l), and TE-saturated phenol was added (40  $\mu$ l; Appendix A.2.3). After vortexing briefly, the phenol was completely removed by extracting several times

with water-saturated ether. The DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M sodium acetate (pH 4.8), and 2 volumes of 95% ethanol, cooling to  $-70^{\circ}\text{C}$  for 5 min, and centrifuging for 30 min in a microfuge at  $4^{\circ}\text{C}$ . After centrifugation the pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

#### A.1.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-borate EDTA (TBE) or Tris-acetate EDTA (TAE) buffers (Appendix A.2.3) were used routinely. Sigma type II agarose was used at varying concentrations (0.8%-1.2%). The amount of DNA loaded per lane also varied with the sizes and number of fragments, but under normal circumstances about 300 ng of plasmid DNA was used. The gels were electrophoresed at 2 V per cm for 16 h. Gels were stained in electrophoresis buffer containing EtBr ( $0.5\ \mu\text{g}/\text{ml}$ ) for 15-30 min. DNA bands were visualized using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used.

DNA fragments were sized according to standard curves prepared by plotting the mobility against the log molecular mass of DNA fragments of known mass. Standard DNA fragments were obtained by the restriction endonuclease digestion of  $\lambda$  DNA with *Pst*I, *Hind*III or *Eco*R1.

### A.1.7 DNA ligation reactions

DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions when subcloning insert fragments into vectors (use 5-15 pmole DNA/ml). DNA concentration was calculated using the formula  $1 \text{ pmole} = (0.662 \times \text{kb}) \mu\text{g}$ . Vector and insert DNA were added to the ligation reactions at a molar ratio of 1:2.

Ligation reactions containing DNA, ligation buffer (Appendix A.2.3), ATP (Appendix A.2.3) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15°C overnight using 0.1-0.25 units of ligase, whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100 x more ligase.

### A.1.8 Rapid subcloning protocol using gel purification

The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (0.8%) (Seaplaque<sup>R</sup>) in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70°C for 5 min in a microfuge tube and the required amounts (2  $\mu\text{l}$  vector DNA, 8  $\mu\text{l}$  insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ATP, ligase and water (10  $\mu\text{l}$ ). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70°C for 5 min, and then diluted with 4 volumes of TSB solution (Appendix A.2.3).

### A.1.9 The preparation and transformation of competent *E. coli* cells

*E. coli* cells were made competent for DNA uptake according to the method of Chung and Miller (1988). A 1/100 dilution of an overnight *E. coli* culture in LB was inoculated into 25 ml prewarmed LB and incubated at 37°C, with shaking, until the culture had reached early exponential phase ( $A_{600}=0.3-0.6$ ) (2-4 h). The cell culture was poured into a pre-cooled sterile SS34 tube and the cells were harvested at 1000 x g for 5 min at 4°C. The cell pellet was resuspended in 2.5 ml (1/10 volume) ice-cold transformation and storage buffer (TSB) (Appendix A.2.3) and held on ice for 10 minutes. The *E. coli* cells (100  $\mu$ l) were then mixed with DNA (routinely 50 ng) and held on ice for a further 30 min. TSB solution (0.9 ml) containing glucose (20 mM) was added to each transformation mixture and incubated at 37°C for 60 min, to allow expression of the plasmid borne antibiotic marker.

Unused cells could be stored at -70°C after rapid freezing in a dry ice/ethanol bath or liquid nitrogen and retained viability provided that the cells were thawed slowly on ice when needed.

### A.1.10 The preparation and transformation of competent *B. subtilis* cells

*B. subtilis* cells were made competent for the uptake of DNA by a modification of the method of Chang and Cohen (1979). A sample (0.1-1 ml) of an overnight culture of *B. subtilis* was diluted into 40 ml 1 x penassay broth (Appendix A.2.1) and shaken well at 37°C until the cells reached midlog ( $A_{600}=0.4$ ). The cells were harvested using a benchtop centrifuge at 2 600 x g for 5 min, and resuspended in 4 ml SMMP solution (Appendix A.2.3) containing lysozyme (2 mg/ml). The cell suspension was incubated with gentle shaking at 37°C for 1-2 h. When protoplasts were visible microscopically, they were pelleted using a benchtop centrifuge at 2000 x g for 7.5 min. The supernatant was carefully poured off and the protoplasts resuspended in 5 ml SMMP. The protoplasts were pelleted as before and resuspended in 4 ml SMMP. These protoplasts were stable for several hours at room temperature. For DNA transformation 0.5 ml of the protoplast suspension and 0.5 ml of SMM PEG solution (Appendix A.2.3) were added to DNA (100 ng) and gently mixed

for 1-2 min. The transformed cells were plated onto DM3 protoplast regeneration agar plates (Appendix A.2.1) containing antibiotic and incubated at 37°C.

#### A.1.11 Nucleotide sequencing

**PRIMER ANNEALING REACTION:** The supercoiled DNA (6-10 µg, in TE buffer) was diluted to a final volume of 20 µl in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 µl of 3 M sodium acetate (pH 5.2), 25 µl of distilled water and 150 µl of chilled ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 µl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 µl of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM MgCl<sub>2</sub>; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 30 min at 40°C immediately prior to sequencing. The forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 reverse sequencing primer (Amersham) were used, unless otherwise stated.

**SEQUENCING REACTIONS:** DNA sequencing was done by the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a Sequenase sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [ $\alpha$ -<sup>35</sup>S]dATP (1200 Ci/mmol; Amersham).

**GEL ELECTROPHORESIS AND AUTORADIOGRAPHY:** The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2mm thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing <sup>35</sup>S-labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

### **A.1.12 DNA/RNA alkali blotting procedure**

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond N+ hybridization membrane (Amersham) essentially by the protocol of Reed and Mann (1985). The use of a nylon transfer membrane allows the capillary transfer of DNA restriction fragments in alkali rather than in neutral, high ionic strength solvents (used in conventional Southern transfer), and eliminates the need for post-transfer fixation (Reed and Mann, 1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25 M) for 20 min at room temperature with gentle agitation, followed by a brief rinse in distilled water. The gel was then placed on top of 2 sheets of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of an inverted gel-casting tray in a plastic box, such that the filter paper touched the base of the box, forming a wick), and was flooded with 50-100 ml of 0.4 N NaOH. A sheet of Hybond N+ (wetted by floating onto and then immersion in distilled water) was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3 MM filter paper, wetted in 0.4 N NaOH, were laid onto the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue overnight. After transfer, the membrane was rinsed briefly with gentle agitation in 2 x SSC (Appendix A.2.3). The membrane was now ready for hybridization or could be wrapped in saran wrap and stored at 4°C.

### **A.1.13 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)**

**SDS-PAGE PROCEDURE:** Discontinuous SDS-PAGE was done according to the method of Laemmli (1970) and O'Farrell (1975) using a Hoefer SE600 vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Fransisco, CA, USA). The 1.5 mm thick gel spacers were used. The resolving gel was prepared and degassed before pouring. Propan-2-ol was layered on the gel to promote a sharp interface. After the gel had polymerized (30 min), the propan-2-ol was removed by rinsing with the stacking gel buffer, and the stacking gel was cast.

Samples were prepared in sample treatment buffer and placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant current) per gel until the dye front migrated to the end of the gel (four to five hours).

After electrophoresis the gels were stained for 3 h in coomassie blue staining solution with gentle agitation, destained and dried.

The acrylamide resolving gels (12% and 20%) and stacking gels (4%) were prepared as follows:

Solution	Resolving gel		Stacking gel
	20%	12%	
Acrylamide solution	20 ml	12 ml	2.66 ml
Resolving gel buffer	7.5 ml	7.5 ml	-
Stacking gel buffer	-	-	5 ml
SDS	0.3 ml	0.3 ml	0.2 ml
Distilled water	2.0 ml	10.0 ml	12.2 ml
Ammonium persulphate	150 $\mu$ l	150 $\mu$ l	100 $\mu$ l
TEMED	10 $\mu$ l	10 $\mu$ l	40 $\mu$ l

#### SDS-PAGE SOLUTIONS:

##### Acrylamide solution

Acrylamide 29.2 g  
 Bis-acrylamide 0.8 g  
 Distilled water to 100 ml  
 Filter through Whatman's paper (No. 1) and store in dark bottle at 4°C.

##### Resolving gel buffer

Tris (1.5 M) 18.17 g  
 Distilled water to 100 ml  
 Adjust pH to 8.8 (approximately 3.3 ml conc. HCl).

Stacking gel buffer

Tris-HCl (0.5 M)	6.06 g
Distilled water	to 100 ml

Adjust pH to 6.8 (approximately 5.5 ml conc. HCl).

Ammonium persulphate (10% w/v)

A fresh solution was made immediately before use.

Reservoir buffer

Tris base (0.025 M)	15.15 g
Glycine (0.192 M)	72.05 g
10% SDS (0.1% w/v)	50 ml
Distilled water	to 5000 ml

The pH should be approximately 8.5.

SDS (10%)

SDS	50 g
Distilled water	to 500 ml

Coomassie blue staining solution

Coomassie blue R250 (0.25% w/v)	2.5 g
Destaining solution	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No. 1).

Destain solution

Acetic acid	250 ml
Methanol	750 ml
Distilled water	1500 ml

Sample treatment buffer

Stacking gel buffer	2.5 ml
SDS (10%)	4 ml
Glycerol	2 ml
2-mercaptoethanol	1 ml
Distilled water	0.5 ml

The solution was stored in aliquots at -20°C.

## A.2 MEDIA, BUFFERS AND SOLUTIONS

All media, buffers, and solutions were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 µm membrane filters (Millipore). SDS-PAGE solutions and buffers are given in Appendix A.1.13.

### A.2.1 Media

#### CMM SALTS (10 X):

K <sub>2</sub> HPO <sub>4</sub>	121.9 g
KH <sub>2</sub> PO <sub>4</sub>	40.8 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	33 g
Tri-sodium citrate	0.22 g
Distilled water	to 1000 ml

#### CMM BROTH:

10 x CMM Salts	100 ml
Distilled water	900 ml

This salts solution was autoclaved separately and the following sterilized solutions were added:

Carbon source (20%, w/v)	100 ml
MgSO <sub>4</sub> (0.5 M)	1 ml
MnSO <sub>4</sub> (0.01 M)	1 ml
(NH <sub>4</sub> )Fe(SO <sub>4</sub> ) <sub>2</sub> (22 mg/ml)	1 ml
L-amino acids (20 mg/ml)	5 ml

Where necessary this minimal medium was supplemented with vitamin-free casamino acids (Difco Laboratories) at a final concentration of 0.25% (w/v).

#### CMM AGAR:

Agar	15 g
Distilled water	600 ml
Autoclaved separately	
10 x CMM salts	100 ml
Distilled water	300 ml
Autoclaved separately	

All sterilized solutions listed for the CMM broth were added.

#### CSH MM SALTS (5 x):

K <sub>2</sub> HPO <sub>4</sub>	52.5 g
KH <sub>2</sub> PO <sub>4</sub>	22.5 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5 g
Tri-sodium citrate	2.5 g
Distilled water	to 1000 ml

**DM3 AGAR (PROTOPLAST REGENERATION MEDIUM FOR BACILLUS):**

Agar	6 g
Distilled water	200 ml

This solution was autoclaved separately and the following sterilized solutions were added:

Na succinate pH 7.3 (1 M)	500 ml
Casamino acids (5%, w/v)	100 ml
Yeast extract (10%, w/v)	50 ml
K <sub>2</sub> HPO <sub>4</sub> (3.5%, w/v)	50 ml
KH <sub>2</sub> PO <sub>4</sub> (1.5%, w/v)	50 ml
Glucose (20%, w/v)	25 ml
MgCl <sub>2</sub> (1 M)	20 ml
Bovine serum albumin (BSA) (2%, w/v)	5 ml

**LURIA-BERTANI MEDIUM (LB):**

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

Solid media contained 1.5% (w/v) agar.

**MacCONKEY AGAR:**

MacConkey base (Difco Laboratories)	40 g
Distilled water	to 1000 ml

After autoclaving, 40 ml of a sterilized solution of the carbon source (25%, w/v) was added.

**MM BROTH:**

5 x CSH MM salts	200 ml
Distilled water	800 ml

This salts solution was autoclaved separately and the following sterilized solutions were added:

Carbon source (20%, w/v)	10 ml
MgSO <sub>4</sub> (20%, w/v)	1 ml
Vitamin B1 (1%, w/v)	0.5 ml
L-amino acids (20 mg/ml)	1 ml
NaCl (25%, w/v)	20 ml
Vitamin-free casamino acids (Difco Laboratories) (25%, w/v)	10 ml

**MM AGAR:**

Agar	15 g
Distilled water	600 ml
Autoclaved separately	
5 x CSH MM salts	200 ml
Distilled water	200 ml
Autoclaved separately	

All sterilized solutions listed for MM broth were added with the exception of NaCl and vitamin-free casamino acids, which were omitted.

**PENASSAY BROTH (4 x):**

Beef extract	6 g
Yeast extract	6 g
Peptone	20 g
Glucose	4 g
NaCl	14 g
K <sub>2</sub> HPO <sub>4</sub>	14.4 g
KH <sub>2</sub> PO <sub>4</sub>	5.28 g
Distilled water	to 1000 ml

**A.2.2 Media Additives**

Media were cooled to 50°C before addition of antibiotics, cAMP or XP. Plates containing these additives were stored for no longer than one week at 4°C.

**ANTIBIOTICS:**

Antibiotic stock solutions were as follows:

Ampicillin	25 mg/ml water
Chloramphenicol	35 mg/ml ethanol (96%)
Kanamycin	125 mg/ml water

All antibiotics were filter sterilized and stored at -20°C.

**cAMP (ADENOSINE-3',5'-CYCLIC MONOPHOSPHATE) (0.25 M):**

cAMP	410 mg
Distilled water	5 ml

Adjusted the pH to 7.0 with NaOH (0.1 M) before making up to 5 ml. Filter sterilized and stored at -70°C.

**XP (5-BROMO-4-CHLORO-3-INDOLYL PHOSPHATE):**

XP	80 mg
DMSO	1 ml

The solution was stored at -70°C.

### A.2.3 Buffers and solutions

#### ATP (ADENOSINE 5'-TRIPHOSPHATE) (10 mM):

ATP	30 mg
Distilled water	5 ml

Adjusted the pH to 7.0 with NaOH (0.1 M) before making up to 5 ml. Stored in 100  $\mu$ l aliquots at -70°C. Discarded remainder once defrosted.

#### BRADFORD SOLUTION (Bradford, 1976):

Coomassie Brilliant Blue (G-250)	100 mg
Ethanol (95%)	50 ml

Dissolved, then added 100 ml phosphoric acid (85%). Diluted to final volume of 1 l. Filtered through Whatman GF/C filter paper. Stored in dark bottle.

#### DINITROSALICYLIC ACID SOLUTION (DNS) (Miller, 1959):

3,5 Dinitrosalicylic acid	10.6 g
NaOH	19.8 g
Rochelle salts (Na K Tartrate)	306 g
Phenol	7.6 ml
Na-meta bisulphite	8.3 g
Distilled water	1416 ml

The dinitrosalicylic acid, NaOH, and Rochelle salts were dissolved completely in the water before adding the other constituents and dissolving each in turn. The phenol was melted at 50°C. A 3 ml sample was titrated to the end-point with 5-6 ml HCl (0.1 M) using phenolphthalein as an end-point indicator. However, if less HCl was required then solid NaOH was added to the DNS solution at the rate of 2 g/ml of HCl less than five ml, and the titration repeated. The DNS solution was stored in a dark bottle under N<sub>2</sub>.

#### DNA LOADING SOLUTION (6x):

Bromophenol blue	0.25 g
Sucrose	40 g
Distilled water	to 100 ml

The solution was stored at 4°C.

#### EXONUCLEASE III SHORTENING SOLUTIONS (Henikoff, 1984):

##### Exo buffer

Tris-HCl (1 M, pH 8.0)	660 $\mu$ l
MgCl <sub>2</sub> (0.1 M)	66.4 $\mu$ l
Distilled water	9.27 ml

##### Klenow buffer pH 8.0

Tris-HCl (0.1 M, pH 8.0)	3 $\mu$ l
MgCl <sub>2</sub> (1M)	6 $\mu$ l
Distilled water	20 $\mu$ l

S1 buffer (10x)

KOAc pH 4.6 (3 M)	1.1 ml
NaCl (5 M)	5 ml
Glycerol	5 ml
ZnSO <sub>4</sub>	30 mg

S1 mixture

S1 buffer (10x)	41 µl
Distilled water	259 µl
S1 nuclease (60 units)	1.5 µl

S1 stop

Trisma Base (no HCl)	0.3 M
EDTA (pH 8.0)	0.05 M

**LIGATION BUFFER (10 x) (pH 7.6):**

Tris-HCl (1 M, pH 7.6)	0.66 ml
MgCl <sub>2</sub> (1 M)	66 µl
Dithiothreitol	15.4 mg
Distilled water	0.274 ml

Stored in 50 µl aliquots at -20°C. Discarded remainder once defrosted. ATP was added to the ligation mixtures at a final concentration of 1 mM.

**PHENOL (TE-SATURATED):**

Phenol (200 g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10 x) or until the pH of the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1 x) at -20°C.

**SSC (20 x):**

NaCl (3 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1000 ml

Adjusted the pH to 7.0 with NaOH (10 N). Autoclaved.

**T4 DNA POLYMERASE BUFFER (10 x):**

This buffer was prepared using restriction endonuclease buffer A (Boehringer Mannheim) and adding bovine serum albumin (BSA Pentax Fraction V) to a final concentration of 1 mg/ml. Buffer A has the following composition:

Tris-acetate (pH 7.9)	0.33 M
K-acetate	0.66 M
Mg-acetate	0.1 M
Dithiothreitol	5 mM

After addition of the BSA the buffer was divided into 100 µl aliquots and stored at -20°C.

**TRIS-ACETATE EDTA BUFFER (TAE) (pH 8.0) (50 x):**

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml
Distilled water	to 1000 ml

For Tris-acetate buffered electrophoresis using SeaPlaque<sup>R</sup> low melting point agarose, the addition of EDTA was omitted.

**TRIS-BORATE EDTA BUFFER (TBE) (pH 8.0) (5 x):**

Tris base	54 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20 ml
Distilled water	to 1000 ml

**TRIS-EDTA (TE) BUFFER (pH 8.0):**

Tris base	1.21 g
EDTA (0.5 M, pH 8.0)	2 ml
Distilled water	to 1000 ml

Adjusted the pH to 8.0 with HCl (0.1 M). Autoclaved.

**TRANSFORMATION SOLUTIONS FOR *BACILLUS*:**SMM Buffer (pH 6.5) (2 x)

Sucrose	342.3 g
Maleate	4.6 g
MgCl <sub>2</sub>	8.1 g
Distilled water	to 1000 ml

Adjusted the pH to 6.5 with NaOH (0.1 M). Autoclaved.

SMMP solution (protoplast solution)

Made by mixing equal volumes of 4 x penassay broth and 2 x SMM buffer.

SMM PEG solution (40% PEG 6000)

PEG 6000	40 g
SMM (2 x)	50 ml
Distilled water	50 ml

**TSB SOLUTION:**

LB	150 ml
pH to 6.1 with 2 drops conc. HCl.	
PEG 4000	15 g
MgSO <sub>4</sub> (1 M)	1.5 ml
MgCl <sub>2</sub> (1 M)	1.5 ml

Dispensed in 20 ml aliquots and autoclaved. Added DMSO (1 ml) and glucose (0.5 M, 400 µl when necessary) immediately before use.

## APPENDIX B

## CLONING VECTORS AND TRANSPOSON RESTRICTION MAPS

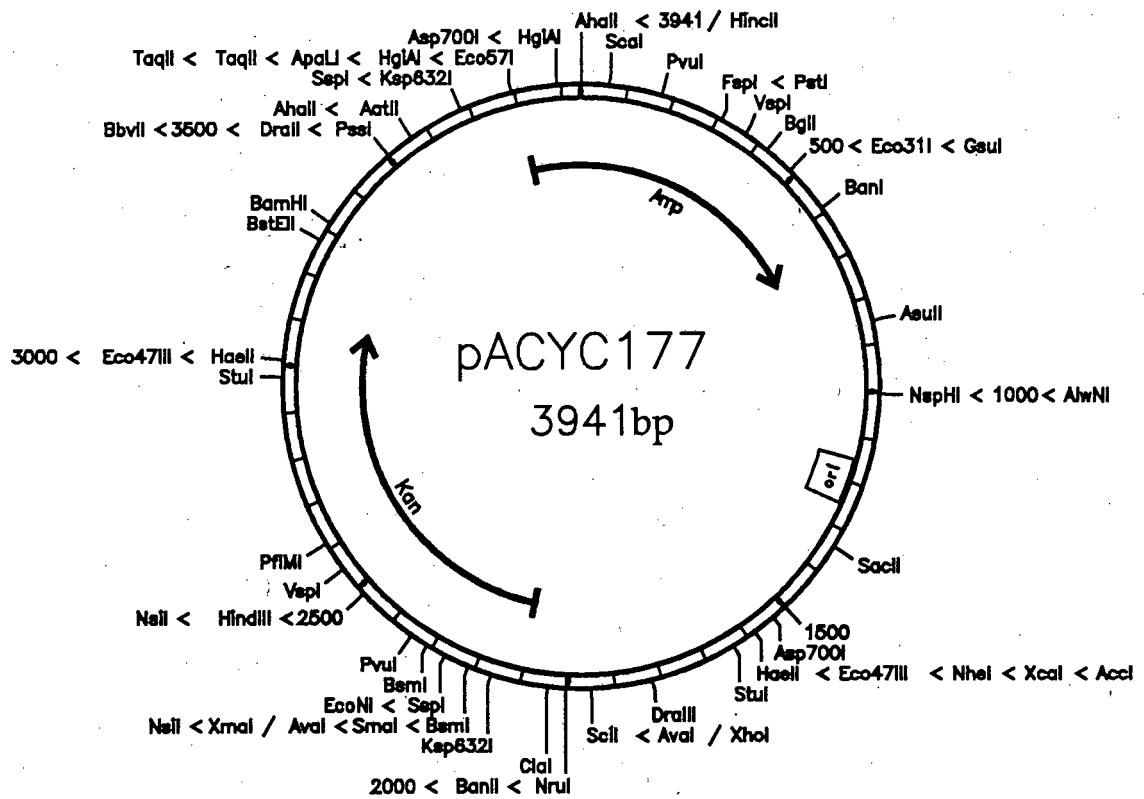


Fig. B.1 Restriction map of pACYC177 (Chang and Cohen, 1978). This map was generated using the PlasmidMap and MapSort programs from the GCG package version 6.1.



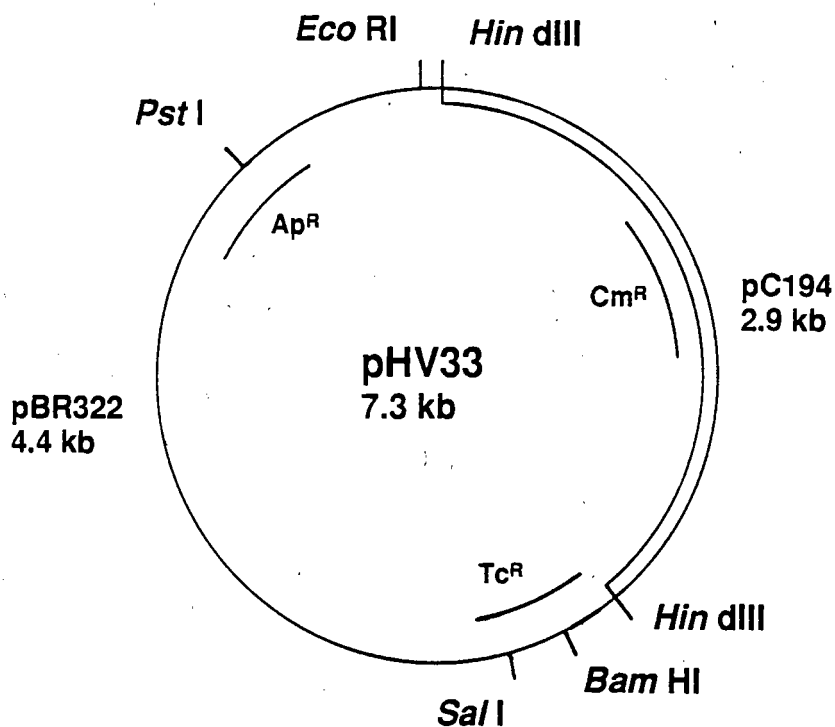


Fig. B.4 Restriction map of pHV33 (Primrose and Ehrlich, 1981). Restriction endonuclease sites are shown for those restriction endonucleases that cleave the molecule once or twice.

### TnphoA

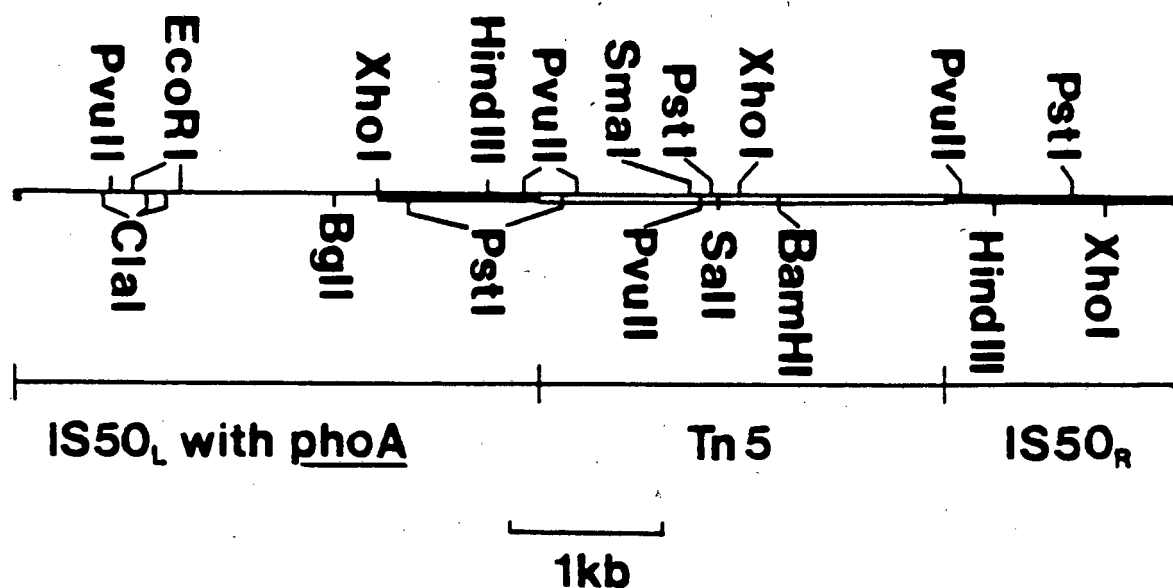


Fig. B.5 Partial restriction map of Tn*phoA* showing the position of Tn5 relative to the leftward and rightward insertion sequences (Manoil and Beckwith, 1985; Gutierrez *et al.*, 1987).

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