STUDIES ON THE *VIBRIO ALGINOLYTICUS* SUCROSE UTILIZATION SYSTEM CLONED INTO *ESCHERICHIA COLI*

by

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Für meinen Vater,

der mir mehr als das war.

Note

Following submission of this thesis, the notation *scrA* has been changed to *scrB* for the *V. alginolyticus* sucrase gene. This was done in order to comply with the nomenclature adopted in the literature describing other bacterial sucrose utilization systems.

CERTIFICATION OF SUPERVISOR

In terms of paragraph 8 of "General Regulations for the Degree of Ph.D" I, as supervisor of the candidate R R Scholle, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Signed by candidate

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ABBREVIATIONS

A	adenine
aa	amino acid(s)
Ala	L-alanine (A)
Ap	ampicillin
Arg	L-arginine (R)
Asn	L-asparagine (N)
Asp	L-aspartic acid (D)
bp	base pair(s)
C	cytosine
cAMP	adenosine-3′,5′-cyclic monophosphate
CAP	catabolite activator protein
Ci	Curie
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
Clm	chloramphenicol
cpm	counts per minute
CsCl	caesium chloride
Cys	L-cysteine (C)
D	Dalton(s)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate(s)
EDTA	ethylenediaminetetra-acetic acid
EtdBr	ethidium bromide
g	standard gravitational acceleration
G	guanine
Gln	L-glutamine (Q)
Glu	L-glutamic acid (E)
Gly	L-glycine (G)
h	hour(s)
His	L-histidine (H)
Ile	L-isoleucine (I)
IPTG	*iso*propyl-thio-β-D-galactoside
Kan	kanamycin
kb	kilobase(s)
kD	kilodalton(s)
LB	Luria-Bernardi
Leu	L-leucine(L)
Lys	L-lysine (K)
Met	L-methionine (M)
min	minute(s)
MM	minimal medium
M_r	relative molecular mass
nt	nucleotide(s)

O	operator
OD	absorbance
ONPG	*o*-nitrophenyl-β-D-galactoside
ORF	open reading frame
p	plasmid
P	promoter
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
Phe	L-phenylalanine (F)
Pro	L-proline (P)
PTS	phosphotransferase system
r	(superscript) resistance
RNA	ribonucleic acid
RNase	ribonuclease
s	second(s)
SDS	sodium dodecyl sulphate
Ser	L-serine (S)
Str	streptomycin
T	thymine
Tet	tetracycline
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
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside
α	alpha
β	beta
Δ	delta
λ	lambda
μ	micro
()	plasmid carrier status

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ABSTRACT

This dissertation forms part of the study on the molecular biology of the aerobic, collagenolytic, halotolerant, gram-negative organism *Vibrio alginolyticus*. The cloning, expression and regulation of the *V. alginolyticus* sucrose utilization system in *Escherichia coli* is discussed and the results of a molecular analysis of the sucrase gene (*scrA*) are presented.

The clone pVS100, containing a sucrose utilization system, was isolated from a genomic library of *V. alginolyticus*. Plasmid pVS100 was mapped and the origin of its insert determined by Southern blotting and DNA hybridization. The number and sizes of the polypeptide products encoded by plasmid pVS100 were determined by DNA-directed cell-free protein synthesis. The capsule, produced by transformed and untransformed *E. coli* JA221 cells, was shown to be independent of the presence of plasmid pVS100. The sucrase activity assay was optimized with respect to time, pH, temperature and salt requirements.

Sucrase activity was detected both intra- and extracellularly in *E. coli* JA221 (pVS100) cultures. In *V. alginolyticus* only intracellular sucrase was detected. Sucrase synthesis in *E. coli* JA221 (pVS100) was induced by sucrose and repressed by glucose, and the repression was reversed by cAMP. The same responses were observed for sucrase synthesis in *V. alginolyticus*. Transformation of pVS100 into *E. coli* mutant strains deficient in the catabolite activator protein (CAP) and adenylate cyclase, confirmed that at least functional CAP is required for the induction of the sucrose utilization system. Localization of sucrase activity in *E. coli* JA221 (pVS100) by osmotic shock indicated that on average 24% of the sucrase activity was variably distributed between the periplasm and the extracellular supernatant fluid. The presence of sucrase in the supernatant was attributed to non-specific leakage across the periplasmic membrane. Results obtained with cytoplasmicly located control enzymes however, ruled out the possibility of protein leakage across the cytoplasmic membrane. Experiments with Tn*phoA* indicated that translocation of sucrase across the cytoplasmic membrane was mediated by a mechanism which did not involve a signal sequence.

V. alginolyticus and *E. coli* JA221 (pVS100) cells were shown to actively transport sucrose by an inducible, Na^+-independent sucrose transport system. Sucrose was translocated both in the presence and absence of Na^+, K^+ and Li^+. The maximum velocity and apparent K_m values of sucrose uptake for the *V. alginolyticus* strain and *E. coli* JA221

(pVS100) were 130 nmol/mg of protein per min and 50 μM and 6 nmol/mg of protein per min and 275 μM, respectively.

The nucleotide sequence of a 2.119 kb DNA fragment containing the *V. alginolyticus* sucrase gene (*scrA*) was determined. The complete amino acid sequence (484 residues) of the sucrase was deduced and significant homology was detected between the sucrase enzymes from *V. alginolyticus* and *Bacillus subtilis*. The *V. alginolyticus* sucrase did not contain a classical N-terminal signal sequence. The codon usage in *scrA* of *V. alginolyticus* was very similar to other *Vibrio* genes and to *E. coli* derived genes. The *V. alginolyticus scrA* gene on the 2.119 kb DNA fragment was expressed from a putative secondary constitutive promoter in *E. coli*.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1. General introduction to *Vibrio alginolyticus*

The gram-negative bacterium *V. alginolyticus* was isolated from raw hides in a study of bacterial strains responsible for leather decay brought about by the degradation of collagen. This aerobic, halotolerant *V. alginolyticus* strain, initially classified as *Achromobacter iophagus*, displayed strong collagenase activity when propagated in the presence of 2.34% (w/v) NaCl under aerobic conditions and was shown to be the cause of leather decay (Woods *et al.*, 1973; Welton and Woods, 1973). A beneficial use of *V. alginolyticus* however, was reported by Welton and Woods (1975) who indicated that *V. alginolyticus* was more suitable for the commercial production of collagenase than *Clostridium histolyticum*, at that time the major industrial source of this enzyme. The process of large-scale collagenase production and purification from *V. alginolyticus* has several advantages over that of *C. histolyticum*. The extracellular collagenase displays a higher specific activity than the *C. histolyticum* enzyme, and it is not associated with a lethal clostridial toxin (Welton and Woods, 1975; Lecroisey *et al.*, 1975; Keil-Dlouha *et al.*, 1976).

Apart from being of industrial interest, collagenase production by *V. alginolyticus* represents an ideal model system for the study of true exoprotein production by a gram-negative bacterium. Initial studies on collagenase production by wild type and mutant strains of *V. alginolyticus* suggested that a complex induction and regulatory mechanism is involved in the enzyme's production. It was shown that collagenase was induced by collagen and peptone and was subject to end-product inhibition and an independently mediated glucose repression. The addition of cyclic nucleotides did not reverse the glucose repression, indicating that collagenase synthesis is not regulated by classical catabolite repression (Robbertse *et al.*, 1978).

The second enzyme system from *V. alginolyticus* which has been studied is the extracellular alkaline proteases (Long *et al.*, 1981). Protease production occurs during the stationary growth phase and is associated with the inactivation of the collagenase. Protease activity is regulated by end-product inhibition and glucose repression, which is not reversed by the addition of cyclic nucleotides. Surprisingly, protease production is stimulated by histidine, which suggests that the histidine utilization system (*hut*) might be involved in the regulation of the alkaline protease (Long *et al.*, 1981; Bowden *et al.*, 1982). The production of both collagenase and alkaline proteases is regulated by temperature and oxygen. Optimal yields of these enzymes are obtained at 30^{o}C and their production is significantly reduced by a temperature shift to 37^{o}C or by the lack of oxygen (Hare *et al.*, 1981).

Through the development of an extremely sensitive SDS-gelatine-PAGE system it was possible to show that six distinct serine proteases were responsible for the extracellular protease activity in *V. alginolyticus* cultures (Hare *et al.*, 1983; Deane *et al.*, 1986). Responses to temperature shifts and the addition of histidine were caused primarily by the regulation of only one (protease 1) of the six different proteases identified. Growth of *V. alginolyticus* in a complex proteinaceous medium resulted in the identification of a seventh exoprotease (protease A) produced by *V. alginolyticus*. Protease A is an alkaline, serine exoprotease, but differs significantly from the six previously reported exoproteases, in that it is Ca^{2+}-dependent, SDS-resistant and has a higher M_r. Protease A is not regulated by temperature, but its production is reduced in the absence of aeration (Deane *et al.*, 1987 a).

The gene encoding protease A was cloned into *Escherichia coli* and was shown to be expressed from a *V. alginolyticus* regulatory region (Deane *et al.*, 1987 b). Protease A was detected in the supernatant of late stationary phase recombinant *E. coli* cultures grown in proteinaceous media containing $CaCl_2$. Although exoprotease activity was only detected during late stationary growth phase (18-24 h), transcription and translation of the exoprotease occurred before 6 h, during exponential growth. The production of extracellular protease A by *E. coli* was not associated with cell lysis.

Protease A is of particular interest to the detergent industry. The activity and stability of the enzyme over a relatively large temperature range and its resistance to SDS

would make it an effective additive to biodetergents (Deane *et al.*, 1987 b).

Although *V. alginolyticus* is a gram-negative bacterium, it resembles gram-positive *Bacillus* strains in a number of respects. Production of true extracellular proteases during the stationary growth phase and rifampin-insensitive proteolytic enzyme production which is subject to endproduct and glucose repression and not relieved by cAMP, are phenomena usually associated with gram-positive bacteria. *V. alginolyticus* further resembles *Bacillus* strains in that the inducible nitrogen catabolic enzymes are not subject to end product inhibition (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 (Bodasing *et al.*, 1985).

In contrast to the nitrogen catabolic enzymes, the glutamine synthetase of *V. alginolyticus* was shown to be regulated in a manner similar to the glutamine synthetase of *E. coli* and other enterobacteria. The structural gene for glutamine synthetase (*glnA*) was cloned in *E. coli*, where it was shown to be regulated by temperature and oxygen (Maharaj *et al.*, 1986). The responses of glutamine synthetase activity to temperature and oxygen in the recombinant *E. coli* were similar to those observed in the *V. alginolyticus* wild type strain (Maharaj *et al.*, 1986). It was speculated that the *V. alginolyticus*-derived *glnA* regulatory regions may also be involved in the regulation of collagenase and exoprotease synthesis. The nitrogen regulatory (*ntr*) genes of gram-negative bacteria regulate other enzyme systems involved in nitrogen metabolism.

1.2. Studies on sucrase enzyme systems

1.2.1. The sucrase enzyme from *Bacillus subtilis*

The sucrase from *B. subtilis* is the most extensively studied sucrase enzyme. The first report of a *B. subtilis* sucrase without associated levan or dextran synthesizing activity was published in 1969 by Prestidge and Spizizen. The characteristics of the sucrase enzyme were shown to differ significantly from those previously reported for the *B. subtilis* levansucrase. In contrast to the levansucrase, sucrase activity was completely intracellular, glucose repressed, induced by comparatively low sucrose concentrations and labile in the absence of added EDTA. The role of the carbon source in induction and repression suggested that the two enzymes were subject to different genetic control mechanisms. These observations were confirmed by Pascal *et al.*, (1971), who identified additional distinguishing features between the levansucrase and sucrase enzymes from *B. subtilis*. The sucrase enzyme was classified as a fructofuranosidase and was shown to be sensitive to trypsin and inactivated by sulfhydryl group reagents, treatments which did not affect levansucrase. Furthermore, sucrase enzyme preparations did not cross-react with antiserum directed against levansucrase and the authors were able to separate the two sucrase activities by column chromatography. The observed differences between the sucrase and levansucrase excluded the possibility of an interconversion between two forms of the same enzymatic protein.

Lepesant *et al.*, (1972; 1976) proposed a tentative pathway for sucrose metabolism in *B. subtilis* (Fig. 1.1) in view of previous reports suggesting that sucrose transport was mediated by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS).

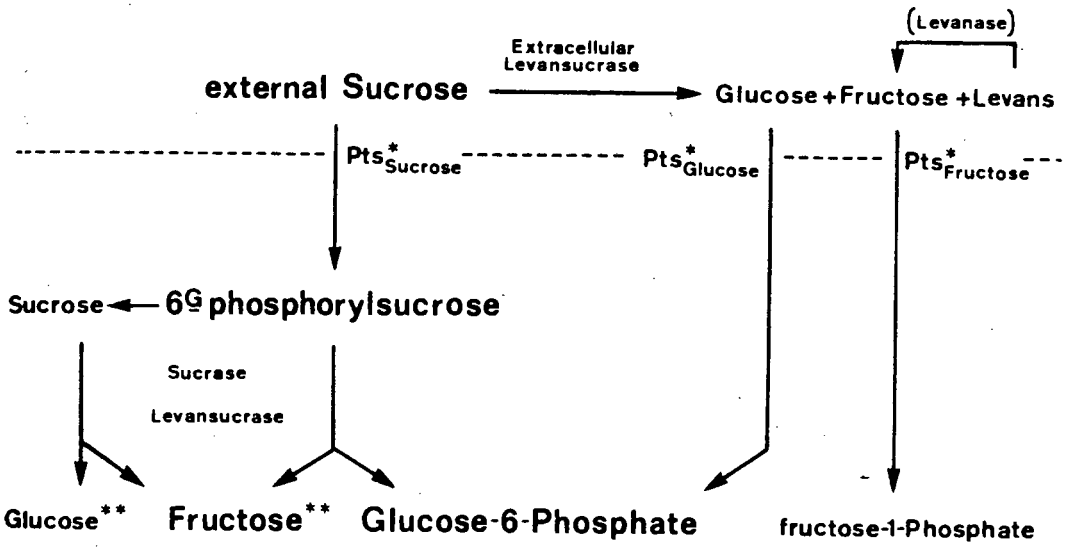

FIG. 1.1. Pathway of sucrose metabolism in *B. subtilis*. One asterisk indicates the reactions catalyzed by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Two asterisks indicate the subsequent metabolism of intracellular glucose and fructose which involves phosphorylation by specific kinases or, in the case of fructose, exit followed by phosphorylation by the fructose PTS. (After Lepesant *et al.*, 1976).

Extensive studies on the chromosomal location of mutations affecting sucrose metabolism in *B. subtilis* has led to the identification of seven loci. Structural genes for sucrase (*sacA*) and levansucrase (*sacB*), regulatory genes for sucrase (*sacT*) and levansucrase synthesis (*sacU*, *sacQ*, *sacR*) and a regulatory gene controlling both sucrase and levansucrase synthesis (*sacS*) were located on the *B. subtilis* chromosome. An eighth locus (*sacP*), located on

the same operon as *sacA* was identified by Lepesant *et al.*, (1974 a) and appeared to code for a component of the phosphorylating sucrose transport system. Moreover, evidence has been obtained that a third saccharolytic enzyme is synthesized by *B. subtilis*. Mutants were isolated which constitutively produced an enzyme exhibiting a broad substrate specificity and which was antigenically unrelated to sucrase and levansucrase. This enzyme was called levanase and is encoded by a gene designated *sacC* (Lepesant *et al.*, 1976; Martin *et al.*, 1987 b). The contribution of this enzyme to sucrose metabolism under normal physiological conditions appears to be limited and the regulation of its synthesis seems to be distinct from that of the other enzymes of the sucrose pathway (Section 1.3.2).

Mapping studies indicated that the loci designated *sacA*, *sacP*, and *sacT* and the loci *sacB* and *sacR* are closely linked in two separate groups on the *B. subtilis* chromosome (Fig. 1.2).

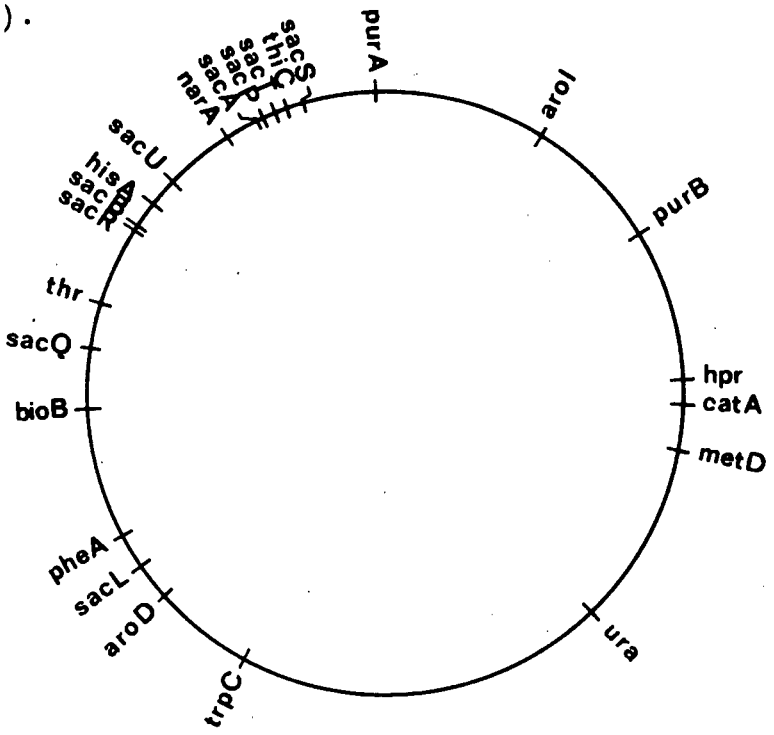

FIG. 1.2. Simplified genetic map of the *B. subtilis* chromosome. (After Lepesant *et al.*, 1976).

A tentative model of the regulation of *sacA*, *sacP* and *sacB* has been proposed by Lepesant *et al.*, (1976) and is depicted in Fig. 1.3.

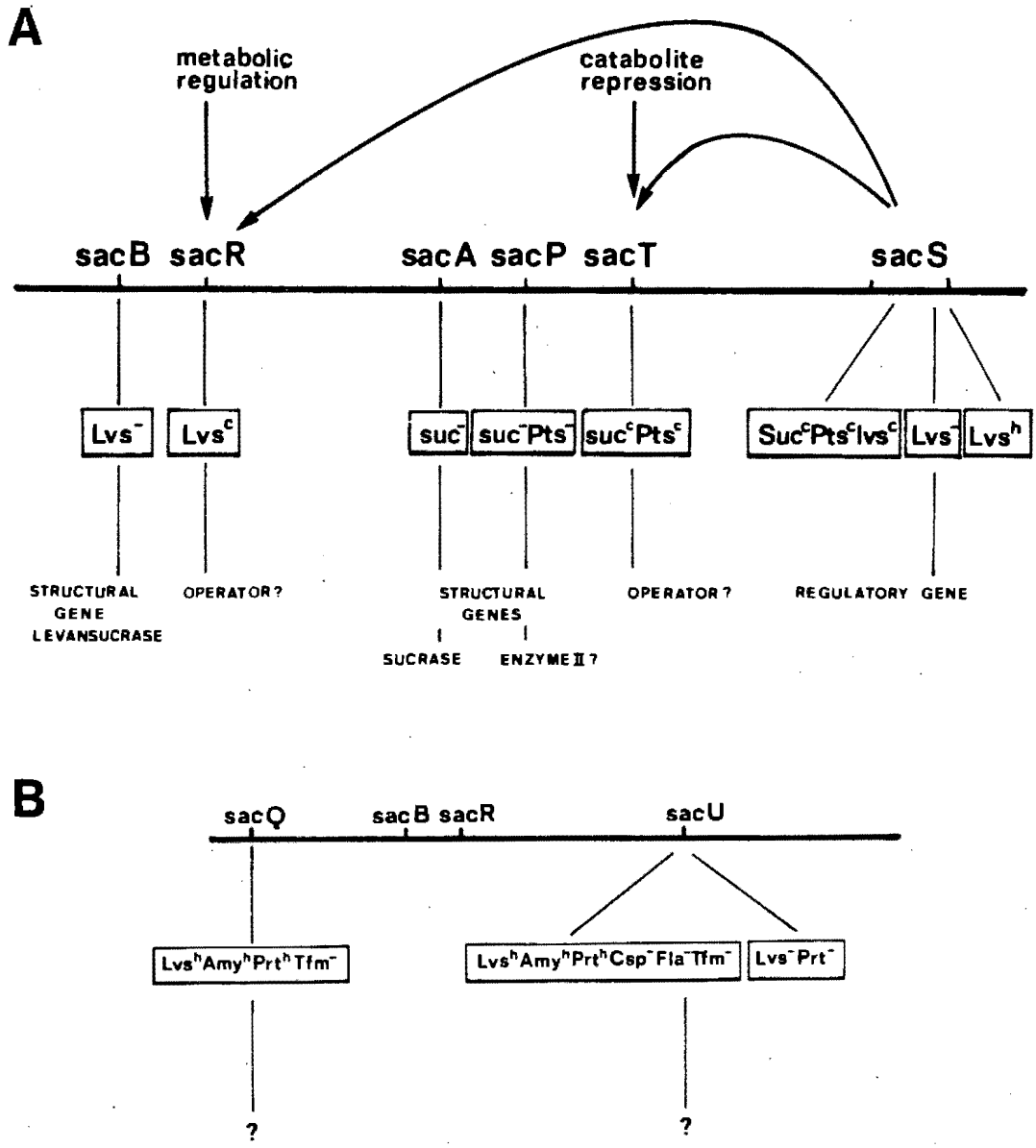

FIG. 1.3. Summary of the phenotypes of the mutations defining the structural and regulatory genes of the sucrose system (A) and the *sacU* and *sacQ* genes (B). Abbreviations: Lvs, levansucrase; Suc, sucrase; Prt, exocellular serine and metal proteases; Amy, α-amylase; Fla, flagella; Tfm, competence 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).

In this model, the *sacS* gene was considered a common regulatory gene to both groups of structural genes (Fig. 1.3 A). The product of the *sacS* gene was presumed to bind the inducer molecules and to control the expression of the *sacA*, *sacB* and *sacP* genes by acting on *sacT* and *sacR*.

Mutations in the genes *sacQ* and *sacU* did not affect the synthesis of the *sacA* and *sacP* products but appeared to affect the control and expression of the levansucrase only (Fig. 1.3 B and Section 1.3).

Growth of *B. subtilis* in media containing low concentrations of sucrose appeared to be largely or completely supported by the endocellular sucrase activity as opposed to the extracellular levansucrase activity. It was shown that the generation time of a sucrase$^+$-levansucrase$^-$ *B. subtilis* mutant was unaltered when compared to the generation time of the wild type strain propagated in sucrose medium. The generation time of levansucrase$^+$-sucrase$^-$ *B. subtilis* mutants, however, was increased significantly under the same experimental conditions (Kunst *et al.*, 1974). Purified sucrase enzyme consists of a single polypeptide with an apparent M_r of 55 000 and displays a pH optimum of 6.5 (Kunst *et al.*, 1974).

The entire *sacA* and part of the *sacP* gene from *B. subtilis* was cloned into *E. coli* (Fouet *et al.*, 1982). Expression of the *sacA* gene cloned into pQB79-1 was shown to remain sucrose inducible in transformed *B. subtilis sacA*$^-$ mutants, and complemented the *sacA* mutation on the *B. subtilis* chromosome. Transformation of *E. coli* with the subcloned *sacA-sacP* containing recombinant vector pHV33

resulted in the production of a functional, and constitutively produced, sucrase enzyme. Despite expression of the heterologous *sacA* gene in *E. coli*, its production did not mediate bacterial growth on minimal media containing sucrose as the sole source of carbon. It was considered that the lack of significant sucrose uptake by the recombinant *E. coli* cells was responsible for the cryptic expression of the *B. subtilis* sucrase. Sequence information obtained from the *sacA-sacP* clone confirmed previous results which suggested that *sacA* and *sacP* were located on the same operon. The deduced amino acid sequence of *sacA* revealed no significant homology to the amino acid sequence of the *B. subtilis* levansucrase, suggesting that the two saccharolytic enzymes from *B. subtilis* were not derived from a common ancestral gene. Strong homology was detected, however, between the *B. subtilis* sucrase and the yeast invertase (Fouet *et al.*, 1986; Carlson and Botstein, 1982; Taussig and Carlson, 1983; Section 1.3).

1.2.2. The sucrase enzymes from members of the genus *Streptococcus*

Streptococcus mutans and closely related oral streptococci are regarded as the principal agents of human dental caries. The bacteria cause considerable damage by virtue of their abilities to adhere to teeth and to produce metabolic acid from fermentable dietary carbohydrates. Sucrose is the predominant carbon source implicated in dental decay (Gustafsson *et al.*, 1954; Hamada and Slade, 1980). Sucrose-dependent pathogenesis by *S. mutans* is two-

fold in that both extended adherence to smooth dental surfaces and subsequent demineralization of tooth material are direct consequences of sucrose catabolism by the organism. Sucrose-mediated adherence of *S. mutans* is dependent on the synthesis of a glucan polymer from sucrose by extracellular glucosyltransferases (Section 1.3.4). The causative relationship between dental caries and streptococci has brought about an extensive study on the sucrose metabolism of these bacteria. It should be noted that the discussion of the sucrose metabolism of streptococci in this and the following sections is by no means exhaustive, and that merely a few interesting results are emphasized.

Thompson and Chassy (1981) have purified a sucrose-inducible, intracellular sucrose-6-phosphate hydrolase from *Streptococcus lactis* K1. Sucrose-6-phosphate hydrolase is responsible for the cleavage of sucrose-6-phosphate (the intracellular product of a sucrose-PTS), yielding glucose-6-phosphate and fructose. The apparent M_r of sucrose-6-phosphate hydrolase was 28 000 and sucrose was found to be a competitive inhibitor of sucrose-6-phosphate hydrolysis. The closely related sucrose-6-phosphate hydrolase encoded by gene *scrB* on the *S. mutans* chromosome was cloned into *E. coli* by Lunsford and Macrina (1986). Sucrose-6-phosphate hydrolase activity was demonstrated in the recombinant *E. coli*, which produced an additional polypeptide of an apparent M_r of approximately 57 000. Sucrose and sucrose-6-phosphate served as substrates for the sucrose-6-phosphate hydrolase of *S. mutans*. In contrast to the *scrB* gene

product synthesized by the recombinant *E. coli*, the intracellular invertase from wild type *S. mutans* had a lower apparent M_r of approximately 48 000. The reason for this difference in M_r was not established but might have represented a posttranslational proteolytic event which occurred in *S. mutans* but not in the recombinant *E. coli*. A third sucrase, from *Streptococcus salivarius*, was cloned and expressed in *E. coli* (Houck *et al.*, 1987). The gene encoding this sucrase enzyme displayed no significant DNA homology to the yeast invertase clone as determined by DNA-DNA hybridization studies.

1.2.3. The sucrase enzymes from members of the genera *Salmonella* and *Klebsiella*

In contrast to the gram-positive bacteria, comparatively few sucrase enzyme systems of gram-negative bacteria have been studied. Among the *Enterobacteriaceae*, only the *Klebsielleae* are characterized by a stable sucrose-positive phenotype. For the other genera, including the closely related *Vibrionaceae*, an unstable sucrose phenotype is typical. The genes for such variable metabolic properties seem to be located on (transmissible) plasmids of unknown origin (Lengeler *et al.*, 1982). A conjugative plasmid, pUR400, isolated from a clinical sucrose-positive *Salmonella typhimurium* strain enabled *E. coli* transformants to utilize sucrose as the sole source of carbon (Schmid *et al.*, 1982). Two inducible plasmid-encoded functions were shown to be involved in sucrose metabolism: (i) a sucrose-specific transport system (Section 1.4) and (ii) an

intracellular sucrose-6-phosphate hydrolase. These functions were encoded by *scrA* and *scrB*, respectively and appeared to be located in a *scr* operon with the gene order *scr* (O, P) *scrA scrB*. Simultaneous induction and catabolite repression of the *scrA* and *scrB* genes provided supportive evidence for this hypothesis. The product of a third, unlinked gene, *scrR*, seemed to exert negative control on the expression of the *scr* structural genes. One of the two differentiating characteristics of the sucrose-6-phosphate hydrolase from *S. typhimurium* in comparison with the sucrose-6-phosphate hydrolases described in the previous section, was the fact that fructose, or a metabolite thereof, functioned as the endogenous inducer. The active sucrose-6-phosphate hydrolase from *S. typhimurium* was furthermore shown to be a 110 000-dalton dimer consisting of 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 subclone pSE64 appeared to contain the three genes described above, *scrA*, *scrB* and *scrR*, which encoded polypeptide products of apparent M_r of approximately 25 000, 60 000 and 41 000 as shown by *E. coli* maxicell analysis. Results obtained were compatible with those from

Schmid *et al.*, (1982, see above) suggesting that the *scrA* and *scrB* genes were located on one operon, however the gene order appeared to be *scr* (O, P) *scrB scrA* as opposed to the previously reported order *scr* (O, P) *scrA scrB*. No correlation has been observed between the partial restriction maps of the *B. subtilis* and *S. typhimurium* sucrase genes (Fouet *et al.*, 1982; Garcia, 1985).

In an attempt to clarify the discrepancy of the gene order, Schmid *et al.*, (1988) reinvestigated the molecular structure of the *S. typhimurium* sucrose system. The *scr* genes of the catabolic plasmid pUR400 were subcloned into pBR328 and analysed in *E. coli* K12. *E. coli* harbouring the resulting recombinant plasmid displayed a sucrose-positive phenotype established by sucrase and sucrose-transport activities which were inducible by sucrose and its breakdown product fructose. The proteins encoded by the *scr* genes were analysed in an *E. coli* minicell system and identified as follows (gene products and apparent M_r are given in brackets): *scrA* (sucrose transport protein, 45 000), *scrB* (invertase, 55 000), *scrR* (repressor protein, 37 000), *scrK* (unknown function, 39 000), and *scrY* (unknown function, 58 000). The genes were shown to be arranged in the order *scr* (*K*, *Y*, *A*, *B*, *R*) and transcribed in the direction from *K* to *B*. 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 obtained by Garcia (1985). The four genes *scr* (*K*, *Y*, *A* and *B*) were shown to be under the control of the *scrR* gene product. In *scrR*-positive cells, expression of the four structural genes was inducible by sucrose but

was constitutive when *scrR* was deleted. The gene product of *scrK* appeared to be non-essential for sucrose metabolism in *E. coli* K12. Preliminary results indicated that the gene product of a M_r of 39 000 was a cytoplasmic, ATP-dependent fructokinase similar to one involved in the sucrose metabolic pathway of *Klebsiella pneumoniae*. The physiological role ascribed to this fructokinase was the phosphorylation of intracellular fructose derived from the hydrolysis of intracellular sucrose-6-phosphate (Sprenger and Lengeler, 1988). The loss of this kinase still allowed growth, although at a reduced rate. A possible function assigned to the *scrY* protein was that it acts as an outer membrane sucrose-specific porin required in particular during growth in low sucrose concentrations.

The chromosomally encoded sucrose utilization system from *K. pneumoniae* displayed striking similarities when compared to that of *S. typhimurium* encoded by plasmid pUR400 (Sprenger and Lengeler, 1988). Both systems are of a sucrose-PTS type, involving a soluble invertase (*scrB*) and fructokinase (*scrK*) and a sucrose-specific transport system mediated by the gene product of *scrA* which required the expression of gene *crr* for full activity (Section 1.4). The structural genes of both systems appear to be clustered in a *scr* regulon which is inducible by fructose or a derivative thereof. The similarities extend to the characteristics of the gene products, which are of identical size and properties, high cross-specificity between repressor/operator pairs and significant homology at the DNA level. These results suggested a recent common origin of the sucrose systems from *K. pneumoniae* and *S. typhimurium*.

1.3. Studies on other saccharolytic enzymes

1.3.1. The levansucrase from *B. subtilis*

The sucrose-inducible, extracellular levansucrase from *B. subtilis* catalyzes mainly the following reaction of transfructosylation:

Sucrose + Acceptor -> Glucose + Fructosyl-acceptor.

Depending on the experimental conditions, water, alcohols, monosaccharides, sucrose, oligosaccharides and levans may act as fructosyl-acceptors. In the presence of sucrose alone, the enzymatic activity leads to the formation of free fructose, oligosaccharides and levans (Chambert *et al.*, 1974). Comparisons of experimental and theoretical results supported a "ping-pong" mechanism, involving the intermediate participation of a covalently linked fructosyl-enzyme complex (Chambert and Gonzy-Treboul, 1976 a; 1976 b). The levansucrase, an elongated, ellipsoid molecule, was shown to consist of a single polypeptide chain with an apparent M_r of 50 000 to 54 000 (Gonzy-Treboul *et al.*, 1975; LeBrun and Van Rapenbusch, 1980; Fouet *et al.*, 1984).

As described in Section 1.2.1, the structural gene for levansucrase was mapped in the *sacB* locus (Lepesant *et al.*, 1974 a; 1974 b). This locus (*sacB*) was cloned into *E. coli* by two groups of workers (Gay *et al.*, 1983; Fouet *et al.*, 1984). In contrast to the results obtained by Fouet *et al.*, Gay *et al.*, detected levansucrase activity only in crude extracts prepared from exponential-phase *E. coli* transformants. The rate of levansucrase synthesis in *E. coli* transformants, relative to the gene copy number, was

comparable to that in the wild type *B. subtilis* reference strain. Fouet *et al.*, 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 β-lactamase activity was detected in the supernatant fluid. Independent nucleotide sequencing of the entire *sacB* gene and the N-terminal 400 basepairs of *sacB* clearly indicated that the intracellular precursor form of levansucrase contained a classical signal sequence of 29 amino acid residues (Fouet *et al.*, 1984; Steinmetz *et al.*, 1985). The precursor form had an apparent M_r of approximately 53 000, which was reduced to a M_r of 50 000 after processing and translocation. The mature levansucrase was identical in both the *E. coli* transformants and the *B. subtilis* reference strain. Analysis of the nucleotide sequence upstream of the *sacB* ATG start codon suggested that the levansucrase was translated from a polycistronic mRNA (Fouet *et al.*, 1984). Reinvestigation of this region led to the identification of a constitutive promoter which was recognized by *E. coli* transformants, and which was separated by 200 basepairs from the translational start codon of *sacB*. This 200 basepair region preceding *sacB* consisted of a short open reading frame (ORF) which included two long complementary invert repeats, a feature characteristic of an attenuator. Several observations made

by Shimotsu and Henner (1986) substantiated the attenuator-hypothesis. Mutations or deletions introduced into this ORF (which was identified as the previously mapped *sacR* locus) lowered the stability of the stem and loop structure of the attenuator which in turn resulted in constitutive transcription of the adjacent *sacB* gene. The ORF was not translated *in vivo*. SI nuclease mapping of the *sacB* transcripts isolated from the wild type *B. subtilis* strain showed that the inducer, sucrose, did not increase the number of transcripts extending into the attenuator structure; however, sucrose did increase the number of transcripts extending past the stem and loop termination structure into the *sacB* structural gene. From these and previous results the following model was proposed: a regulatory molecule, activated by sucrose, binds at or near the stem region of the mRNA termination structure of the nascent transcript, preventing formation of the terminator and allowing transcription of the *sacB* gene. A candidate for such an antitermination regulatory molecule was suggested to be the product of the *sacS* gene, acting *in trans*.

Preliminary characterization of the *sacS* locus suggested that this locus contained two genes, encoding a negative and a positive regulator of *sacB* expression (Aymerich and Steinmetz, 1987; Debarbouille *et al.*, 1987). The negative regulator was proposed to repress the synthesis or the function of the positive regulator and could have been sucrose-dependent. The positive regulator encoded by *sacS*, on the other hand, was the antiterminator acting

either directly or indirectly on the palindromic region encoded by *sacR*.

In section 1.2.1 it was mentioned that mutations in the *sacQ* and *sacU* genes affected the control and expression of levansucrase only, whereas the regulation of the sucrase enzyme remained unaltered. Interestingly, mutations in the *sacQ* and *sacU* genes of *B. subtilis*, apart from affecting the levansucrase, displayed extended pleiotropy indicating that these two genes have a regulatory role in a physiological process of general importance. The phenotypes affected were the synthesis of extracellular serine and metal proteases and α-amylase, sporulation and transformability and the production of flagella. Since all these phenotypes are directly or indirectly associated with cell-membrane functions, it was speculated that the mutations affected a cell envelope-bound function. This speculation appeared unlikely in the case of *sacQ*, however, which was cloned and sequenced by Yang *et al.*, (1986). Although an increased production of the 46 amino acid residue *sacQ* gene product resulted in a concomitant increase in levels of some extracellular proteins, the level of a cytoplasmic protein was elevated as well. Furthermore, interrupting the *sacQ* gene by insertion of a heterologous 1.1 kb DNA fragment did not lead to any detectable phenotypic changes. It therefore appeared unlikely that the *sacQ* gene was required as a component of the secretory machinery.

The *sacU* gene product, on the other hand, initially appeared to be associated with a membrane-bound function. Aubert *et al.*, (1985) reported that the cloned *sacU* gene was

expressed and encoded a polypeptide with an apparent M_r of 46 000 in a minicell producing *E. coli* strain. Membrane fractions of *B. subtilis* sacU protein hyperproducing mutants were characterized by the presence of an increased amount of a 46 kilodalton (kD) protein when compared to the wild type strain. This modification was accompanied by the concomitant disappearance of a 36 kD polypeptide corresponding to the flagellin subunit. The absence of flagella in the sacU protein hyperproducing *B. subtilis* mutant, might therefore have been a consequence of the increased amounts of the 46 kD protein, which could have prevented the normal anchoring of other membrane proteins. It was therefore postulated that the *sacU* gene product may play a role in the mechanism of protein secretion in *B. subtilis*. Two independent groups of workers obtained data contradicting this hypothesis (Shimotsu and Henner, 1986; Aymerich *et al.*, 1986). In contrast to the results obtained by Aubert *et al.*, (1985), it was shown that the gene products of the *sacQ* and *sacU* loci appeared to increase the transcription of *sacB* significantly; in other words these gene products affected the expression of levansucrase at a step prior to the secretion process.

It was further shown that the *sacR* locus was the target of all the identified regulatory genes that affected *sacB* expression (*sacU*, *Q* and *S*). Division of the *sacR* region into two components: (a) a strong, constitutive promoter and (b) the downstream region carrying a palindromic structure, and determining the effect of the *sacU* and *sacQ* gene products on each component, indicated that the *sacQ* and *sacU*

proteins acted on the upstream promoter region of *sacR* in particular (Zukowski and Miller, 1986; Klier *et al.*, 1987). In view of these results it was suggested that the factors coded for by these genes act as transcription activators. Since the positive regulator encoded by *sacS* acted on the downstream palindromic region of *sacR*, it was concluded that *sacB* regulation by (a) *sacU/sacQ* and (b) *sacS* were two independent processes acting on different target sites on *sacR* (Aymerich and Steinmetz, 1987).

A locus, *sacV*, has recently been identified by Martin *et al.*, (1987 a) as the ninth locus involved in the sucrose metabolism of *B. subtilis*. The *sacV* locus, limited to a 0.5 kb DNA fragment, has been shown to stimulate transcription of *sacB* after sucrose induction when present on a multicopy plasmid in *B. subtilis*. The *sacV* locus has been mapped and sequenced and appeared to encode a 64 amino acid peptide. The exact mechanism by which the gene product of *sacV* participates in the stimulation of levansucrase synthesis remains to be elucidated.

1.3.2. The levanase from *B. subtilis*

As mentioned briefly in section 1.2.1, a third saccharolytic enzyme synthesized by *B. subtilis* was identified and named levanase. Initial biochemical and genetic studies on this enzyme indicated that levanase catalyzed the hydrolysis of the fructose polymers levan and inulin. Levanase expression was shown to be subject to catabolite repression, but its expression did not seem to be regulated by the *sacR*, *S*, *T*, *U* and *Q* regulatory loci. In

contrast to levansucrase and sucrase, levanase was not inducible by sucrose; in fact, no inducer is known for levanase. Martin *et al.*, (1987 b) reported the cloning and expression of the structural gene for levanase (*sacC*) in *E. coli*. The *sacC* locus mapped very closely to *sacL* on the *B. subtilis* chromosome (Fig. 1.2). Sequencing data obtained by the same workers confirmed that levanase is synthesized as an intracellular precursor of apparent M_r of approximately 75 000. After removal of a classical signal sequence, the mature extracellular form of levanase has a M_r of 72 000. Translocation into the periplasmic space and concomitant processing was observed in *E. coli* transformants harbouring the *sacC* gene. The N-terminal region of the protein sequence deduced from the *sacC* DNA sequence was shown to display strong homology with the corresponding regions of the sucrase and yeast invertase DNA sequences. In view of other examples of homology between functionally similar proteins of *B. subtilis* and *Saccharomyces cerevisiae*, it was speculated that the N-terminal portion of the *B. subtilis* sucrase and levanase and the yeast invertase were derived from a common ancestral gene.

1.3.3. The invertase from *S. cerevisiae*

Saccharomyces strains carrying any one of the unlinked *SUC*$^+$ genes *(SUC1-SUC7)* produce the sucrose-cleaving enzyme invertase. *SUC*$^+$ yeast strains synthesize two forms of invertase: a secreted, glycosylated enzyme and an intracellular, nonglycosylated enzyme (Carlson and Botstein, 1982). Biochemical and genetic evidence indicated that the

SUC genes were each structural genes for both forms of invertase (Hackel, 1975; Grossmann and Zimmermann, 1979; Carlson *et al.*, 1981; Rodriguez *et al.*, 1981). The protein moieties of the two forms were very similar - each consisted of a dimer of approximately 60 kD subunits - but no precursor-product relationship has been demonstrated. The level of the secreted, glycosylated form was regulated by glucose repression and accounted for most of the invertase activity in derepressed cells. The intracellular, nonglycosylated invertase was synthesized at low levels, which did not change significantly with changes in glucose concentration. Carlson and Botstein (1982) cloned the structural gene for invertase from yeast (*SUC2*). This clone was used to characterize *SUC* RNA species present in cells grown in excess glucose (where invertase synthesis was repressed) and limiting glucose (where invertase synthesis was derepressed). Two *SUC* RNA species (1.8 and 1.9 kb in size) were detected in derepressed cells, which synthesized the secreted, glycosylated form of invertase as well as the intracellular, nonglycosylated form. Only the smaller (1.8 kb) RNA was found in repressed cells, which synthesized only the intracellular, glycosylated invertase. These data suggested that glucose regulation of the amount of the glycosylated form of invertase occurred, at least in part, at the level of RNA synthesis and stability: the presence or absence of the secreted enzyme correlated with the presence or absence of the larger 1.9 kb *SUC* RNA. The two RNA's were shown to differ only at their 5' ends. Both were transcribed in the same direction from the same region, but the longer RNA contained extra contiguous material at its 5'

end. The amino acid sequence of the 'extra material' resembled a signal sequence. These and previous results led to the proposal of the following model by Carlson and Botstein (1982): the 1.9 kb RNA was suggested to encode the entire sequence of the precursor of secreted invertase, and translation of this was initiated at an ATG codon situated at the beginning of the putative signal sequence. The 5' end of the 1.8 kb RNA would be situated within the signal sequence and would therefore lack the translational start codon used for the 1.9 kb RNA. Translation of the shorter RNA would then begin at the next ATG codon, which was found just one codon beyond the *in vitro* cleavage point of the putative secreted-invertase precursor.

1.3.4. The glucosyl- and fructosyl transferases from members of the genus *Streptococcus*

As discussed in section 1.2.2, streptococci, in particular *S. mutans*, have been implicated as the causative agents for dental caries which is partly attributed to sucrose-mediated adherence dependent on the synthesis of a water-insoluble glucan polymer from sucrose, produced by the enzyme glucosyltransferase.

The *gtfA* genes, encoding the glucosyltransferase enzymes from two different serotype *S. mutans* strains have been cloned and expressed in *E. coli* (Robeson *et al.*, 1983; Pucci and Macrina, 1985). *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 both the *S. mutans* wild type strains as well as in the *E. coli*

transformants, the *gtfA* encoded protein appeared to be embedded in the cytoplasmic membrane with the catalytic site orientated towards the exterior. If part of the glucosyltransferase was indeed translocated across the cytoplasmic membrane, this transport would have taken place in the absence of a classical signal sequence and N-terminal modification. The cloned glucosyltransferase was shown to catalyze the formation of fructose and small water-soluble glucan polymers using sucrose as substrate. Pucci and Macrina (1985) have proposed that the *gtfA* gene product may synthesize a primer glucan used by other enzymes in manufacturing water-insoluble glucan, responsible for the adherence of *S. mutans* colonies to teeth surfaces.

Apart from sucrose-6-phosphate hydrolase and glucosyltransferase activities, a third sucrose-metabolizing activity mediated by a fructosyltransferase enzyme has been identified in *Streptococcus* species. Streptococci have at least two types of fructosyltransferase activity distinguished by their catalytic products, being either inulin or levan. *S. salivarius*, in particular, synthesizes two sucrose hydrolyzing enzymes, a sucrase (discussed in Section 1.2.2) and a fructosyltransferase producing a levan. Houck *et al.*, (1987) cloned the gene encoding the fructosyltransferase enzyme into *E. coli* and reported that similar to *S. salivarius* cultures, cultures of the transformed *E. coli* produced a significant fraction of extracellular levansucrase activity. The levansucrase clone from *S. salivarius* displayed no significant DNA homology to the *B. subtilis* levansucrase clone (Section 1.3.1), as determined by DNA-DNA hybridization studies.

1.4. Studies on sugar uptake systems

In gram-negative cells, certain medium-sized molecules (including disaccharides) are translocated across the outer membrane into the periplasmic space through porin channels. Movement of substances across the inner, cytoplasmic, membrane is accomplished by several kinds of mechanisms, which vary in the energy they require:

(a) Simple diffusion. This is a purely physical process that results in the equilibration of the concentration of a compound between two regions separated by a permeable membrane.

(b) Facilitated diffusion. Like simple diffusion, this requires no energy but unlike simple diffusion it requires a specific membrane protein.

(c) Active transport. This is another carrier-mediated process and it is therefore specific, saturable and can be competitively inhibited. In contrast to simple and facilitated diffusion, active transport allows the accumulation of substrate against a concentration gradient, requiring the expenditure of metabolic energy.

(d) Group translocation. This process 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 back across the membrane barrier, and is thus trapped inside the cell. Group

translocation requires energy (expended at the modification step) but is not strictly an active-transport process because the compound is not concentrated in its original form.

Energy required for active transport may be obtained from two sources: ATP, and electrochemical gradients (often referred to as the proton motive force). The two types of potential energy are interconvertible by the membrane ATPase: ATP hydrolysis can establish a membrane potential gradient and, *vice versa*, a potential gradient can drive ATP formation. Substrate translocation energized by the proton motive force is often accomplished by systems termed symports or antiports. These mechanisms mediate the simultaneous transport of two different substrates in the same or opposite direction, respectively, utilizing H^+ as the cosubstrate. Since energized cells have a proton gradient directed towards influx of protons into the cells, H^+ utilized as a cosubstrate is running down the gradient when entering the cell, whereas the substrate (for example the disaccharide lactose which is translocated by a symport) rides the same carrier running against its gradient.

A variety of sugars are transported by group translocation, resulting in the delivery of sugar phosphates into the cytoplasm. These sugars are phosphorylated during the translocation event by protein complexes referred to as the phosphoenolpyruvate-dependent phosphotransferase system (PEP-dependent PTS). Such systems consist of three components: HPr (a histidine-containing phosphocarrier

protein), Enzyme I (EI), and an Enzyme II complex (EII). The three components catalyze the following reactions:

$$\text{P-enolpyruvate} + \text{HPr} \xrightleftharpoons{\text{EI}} \text{Pyruvate} + \text{P-HPr} \quad \text{(Reaction 1)}$$

$$\text{P-HPr} + \text{Sugar} \xrightarrow{\text{EII}} \text{Sugar-P} + \text{HPr} \quad \text{(Reaction 2)}$$

$$\underset{\text{(outside)}}{\text{P-enolpyruvate} + \text{Sugar}} \longrightarrow \underset{\text{(inside)}}{\text{Sugar-P}} + \text{Pyruvate} \quad \text{(Overall Reaction)}$$

Reactions 1 and 2 occur in the cytoplasm and membrane, respectively. The EII complex is specific; that is, different sugars transported by a PTS require different, specific EII complexes. The phosphorylation reaction of the sugar, which occurs during the transport process, is the first step in the metabolism of that sugar, and is thus energetically economical (Lin *et al.*, 1984).

1.4.1. The sucrose transport system in *V. alginolyticus*

Halophilic marine bacteria, which require Na^+ for growth, have been shown to utilize the electrochemical gradient of Na^+ rather than H^+ for the transport of several substrates (Tokuda *et al.*, 1982 a; Tokuda and Unemoto, 1982 b; Dibrov *et al.*, 1986 a; 1986 b; Bakeeva *et al.*, 1986; Tokuda, 1986; MacLeod and MacLeod, 1986). Two different types of pH dependent energetics were shown to be responsible for the generation of the Na^+ electrochemical potential in *V. alginolyticus*. At acidic external pH the Na^+ electrochemical potential was established by a Na^+/H^+ antiport system driven by the proton motive force generated as the immediate result of respiration. The generation of

the Na^+ electrochemical potential, under these conditions, was secondary to the generation of the proton motive force. At alkaline pH, however, the generation of the Na^+ electrochemical potential was a primary process, performed by a Na^+ pump. Therefore, significant differences were observed in the sensitivity of certain energy-linked processes to the proton conductor carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) at acidic and alkaline pHs (Tokuda and Unemoto, 1983). The active transport of amino acids and sucrose, driven by the electrochemical potential of Na^+, was resistant to CCCP at alkaline pH, but not at acidic pH. The authors proposed that H^+ circulation might not be required for the growth of *V. alginolyticus* at alkaline pH, since a sodium motive force took its place for membrane-linked energy requiring reactions.

Closer investigation of the sucrose-inducible, glucose-repressible sucrose uptake system in *V. alginolyticus* confirmed that sucrose uptake specifically required Na^+. The sucrose was accumulated inside the cells without concomitant phosphorylation and was subsequently hydrolyzed into glucose and fructose by an intracellular sucrase. At alkaline pH, sucrose uptake was insensitive to CCCP, which suggested that this uptake reaction was driven by the Na^+ electrochemical potential as opposed to the proton motive force (Kakinuma and Unemoto, 1985).

1.4.2. The sucrose uptake system from *B. subtilis*

Sucrose uptake studies performed on sucrase$^-$ levansucrase$^-$ *B. subtilis* mutants indicated that the sucrose

uptake system was sucrose-inducible, resulting in a 20-fold increase in the initial rate of uptake after induction. The uptake of sucrose led to the intracellular accumulation of two major products; 6^Gphosphorylsucrose and free sucrose. The production of 6^Gphosphorylsucrose has been used as an argument in favour of the participation of a PTS in this reaction. Biochemical analysis confirmed the presence of a multicomponent PTS in *B. subtilis* consisting of two nonspecific, constitutive, soluble proteins EI and HPr as well as an inducible, specific membrane-bound EII. The phenotype of *sacP* mutants suggested that *sacP* (see Fig. 1.2) was the structural gene for a sucrose-specific component of the sucrose PTS, coding for either EII itself or a protein influencing the activity of EII. As mentioned before (Section 1.2.1), *sacP* appeared to be located on the same operon as *sacA* and was under control of the *sacS* gene product acting on the upstream *sacT* locus (Lepesant *et al.*, 1976). The exact function of the *sacP* gene was elucidated by Fouet *et al.*, (1987), by cloning the *sacP* locus into *E. coli*. The recombinant plasmid containing *sacP* was expressed in *E. coli*, which was devoid of sucrose transport and saccharolytic activities, but contained the nonspecific PTS proteins EI and HPr. The *sacP* gene product, encoded on a multicopy plasmid, was shown to be sufficient to allow the transport of sucrose into the *E. coli* cytoplasm using the *E. coli* EI. The initial rate of sucrose uptake in the *E. coli* transformants corresponded to 30-40% of the rate in wild type *B. subtilis* cells. The presence of intracellular sucrose-6-phosphate in *E. coli* transformants confirmed that

sucrose had been phosphorylated during translocation into the cytoplasm. Since it was shown that *E. coli* cells containing *sacP* transport sucrose by way of a PTS, it is thought likely that *sacP* encodes the sucrose-specific EII of *B. subtilis*. The EII^{suc} was probably an integral protein of the *E. coli* cytoplasmic membrane and sucrose presumably diffused through the outer membrane porins into the periplasm. Further evidence for this hypothesis was obtained from the nucleotide sequence of the *sacP* gene (Fouet *et al.*, 1987). The *sacP* locus was indeed located on the same operon as *sacA* and encoded a polypeptide of a M_r of 49 000. A comparison of the deduced amino acid sequence with that of three EII proteins derived from enteric bacteria led to the identification of four regions displaying significant homology. This was not entirely unexpected, since these four proteins share several common properties, such as interactions with the cytoplasmic membrane and the phosphorylated HPr protein, as well as transport and phosphorylation of a sugar.

1.4.3. The sucrose uptake systems from members of the genus *Streptococcus*

Similarly to *B. subtilis*, members of the genus *Streptococcus* take up sucrose by way of a sucrose-inducible PEP-dependent PTS (St. Martin and Wittenberger, 1979; Slee and Tanzer, 1979; Thompson and Chassy, 1981). The intracellular product of the translocation reaction was identified as sucrose-6-phosphate with the phosphate group attached to the C6 of the glucose moiety. The phosphate

donor for this reaction was shown to be PEP, although its immediate glycolytic precursor, 2-phosphoglyceric acid, could substitute for PEP in the absence of NaF. The sucrose-specific PTS was shown to be under separate genetic control to the glucose-specific PTS and was completely inhibited by the trisaccharide raffinose and the disaccharide lactose. All sucrose PTS systems studied in members of the genus *Streptococcus* were shown to be very efficient systems, capable of effectively scavenging low concentrations of sucrose.

1.4.4. The sucrose uptake systems from members of the genera *Salmonella* and *Klebsiella*

Plasmid pUR400 which was shown to confer a sucrose-positive phenotype in *S. typhimurium* and *E. coli* was mentioned in section 1.2.3. Apart from encoding a sucrase (*scrB*), plasmid pUR400 contained the locus *scrA*, encoding a sucrose-specific EII^{scr} (Lengeler *et al.*, 1982). Both genes, *scrA* and *scrB* were fructose-inducible and under common genetic control mediated by the unlinked locus *scrR* (Schmid *et al.*, 1982). Similar to the PEP-dependent PTS described in the previous sections, sucrose transport and concomitant phosphorylation involves the plasmid-encoded membrane-bound sucrose-specific EII^{scr}, and the cytoplasmic proteins EI and HPr, coded for by the chromosomal genes *ptsI* and *ptsH*, respectively. The intracellular product accumulated after translocation of sucrose was identified as sucrose-6-phosphate. In addition to the three proteins described, a third cytoplasmic protein $EIII^{glc}$ was shown to

play a direct role in the uptake and phosphorylation of sucrose. The phosphocarrier protein $EIII^{glc}$, coded for by the chromosomal gene *crr*, is required for the major D-glucose transport system of *E. coli* K-12. In EII mediated phosphorylation, $EIII^{glc}$ has been implicated with catabolite repression as a regulator of internal cAMP levels, as well as with transient repression as a regulator of intracellular hexose phosphate levels.

As described in section 1.2.3, the sucrose metabolic pathways of *S. typhimurium* and *K. pneumoniae* displayed such striking similarities in almost all aspects that a recent common origin of both systems appeared self-evident. Similar to the mechanism described for *S. typhimurium* (pUR400), sucrose uptake and phosphorylation in *K. pneumonia* was mediated by an EII^{scr} protein coded for by the fructose-inducible chromosomal *scrA* locus. Expression of EIII was required for full activity of the sucrose-PTS (Sprenger and Lengeler, 1988).

CHAPTER TWO

CLONING OF THE *V. ALGINOLYTICUS* SUCROSE UTILIZATION SYSTEM IN *E. COLI*

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CLONING OF THE *V. ALGINOLYTICUS* SUCROSE UTILIZATION SYSTEM IN *E. COLI*

Summary: The clone pVS100, containing a sucrose utilization system, was isolated from a genomic library of *V. alginolyticus*. Plasmid pVS100 was mapped and the origin of its insert determined by Southern blotting and DNA hybridization. The number and sizes of the polypeptide products encoded by plasmid pVS100 were determined by DNA-directed cell-free protein synthesis. The capsule, produced by transformed and untransformed *E. coli* JA221 cells, was shown to be independent of the presence of plasmid pVS100. The sucrase activity assay was optimized with respect to time, pH, temperature and salt requirements.

2.1. INTRODUCTION

The ability of *V. alginolyticus* to metabolize sucrose is an important characteristic that is used to distinguish between *V. alginolyticus* and the closely related pathogen, *Vibrio parahaemolyticus* (Shewan and Veron, 1974; Joseph *et al.*, 1982). Studies on the sucrose utilization system of *V. alginolyticus* have been focused primarily on the sucrose transport system (reviewed in section 1.4.1), whereas comparatively little attention has been given to the biochemistry and molecular analysis of the sucrase enzyme of

this bacterium. No reports, beyond those stating that sucrase is an inducible, intracellular enzyme in *V. alginolyticus*, which catalyzes the hydrolysis of intracellular sucrose into glucose and fructose, have been published (Kakinuma and Unemoto, 1985).

The aim of this study was to further characterize the sucrose utilization system of *V. alginolyticus* (consisting of (a) the sucrase enzyme and (b) the sucrose transport system) in both the wild type *V. alginolyticus* strain and in the sucrose-negative *E. coli* strains, with particular emphasis being given to the molecular analysis of the sucrase enzyme.

The first step envisaged in the study of the sucrose utilization system of *V. alginolyticus* was the cloning of this system in *E. coli* so as to allow its study in a sucrose-negative background and to develop a recombinant *E. coli* strain capable of metabolizing sucrose and low grade molasses. The cloning of the *V. alginolyticus* sucrose utilization system and its preliminary characterization are discussed in this chapter.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains and plasmids

V. alginolyticus (Reid *et al.*, 1980; Welton and Woods, 1973; 1975) was used as the source of chromosomal DNA. *E. coli* JA221 F^- *recA*1 *leuB*6 *trpE*5 *hsdR* *lacY* λ^- (Beggs, 1978) was used as the recipient strain. *E. coli* strains LK111, a *lacI*^{q} *lacZ*ΔM15 *lacY*$^+$ derivative of K514 (Zabeau and Stanley, 1982), and YMC11 (*glnA*$^-$ *ntrB*$^-$ *ntrC*$^-$) (Backman *et al.*, 1981) were used as control strains for the studies carried out on the capsule.

Plasmid pEcoR251, a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belgium, is a positive selection vector containing the *E. coli* *Eco*RI endonuclease gene under the control of the bacteriophage λ rightward promoter, the Ap^r gene, and the plasmid pBR322 origin of replication. It was derived from the pCL plasmids described by Zabeau and Stanley (1982). The *Eco*RI endonuclease gene product, expressed at high levels by the λ promoter on pEcoR251, is lethal unless insertionally inactivated, or regulated by plasmid pCI857, which contains a temperature-sensitive λ repressor gene (Remaut *et al.*, 1983). The *Eco*RI gene has a single *Bgl*II cloning site. Plasmids pVP105 (S. Deane, personal communication) and pRM210 (Maharaj *et al.*, 1986) contain the exoprotease A and the glutamine synthetase genes from *V. alginolyticus*, respectively. Plasmid pVS100, containing the sucrose utilization system from *V. alginolyticus* cloned into plasmid pEcoR251, was constructed in this study.

2.2.2. Standard methods, buffers and media

Appendix A lists standard techniques, buffers and media.

2.2.3. Generation of the *V. alginolyticus* genebank

The genebank of *V. alginolyticus* was prepared by Romilla Maharaj (Maharaj *et al.*, 1986). Chromosomal DNA, extracted from *V. alginolyticus*, was partially digested with endonuclease *Sau*3A and fractionated on a sucrose density gradient. Fragments of 4 to 10 kb were ligated with *Bgl*II endonuclease digested pEcoR251 DNA and used to transform competent cells of *E. coli* JA221. Ap^r transformants were selected on LB agar containing 100 μg of Ap per ml.

2.2.4. Isolation and restriction endonuclease mapping of clone pVS100

E. coli JA221 Ap^r transformants were scraped off the LB agar plates (Appendix A.2), washed with sterile saline (0.15 M NaCl) and spread onto MM plates (Appendix A.2) containing Ap (50 μg/ml) and 6 mM sucrose as the sole source of carbon. Colonies isolated from the sucrose MM plates were transferred onto MacConkey indicator plates (Appendix A.2) containing Ap (50 μg/ml) and 30mM sucrose. Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (1981) (Appendix A.1) from the putative sucrose-positive colonies and the presence of insert DNA in the pEcoR251 vector was verified by restriction endonuclease digestion (Appendix A.1). A restriction endonuclease map was constructed by digesting plasmid DNA with a variety of endonucleases and

resolving the digestion products by electrophoresis in TBE buffered 0.8% agarose gels (Appendices A.1 and A.2).

2.2.5. Southern hybridization studies

V. alginolyticus chromosomal DNA, prepared by the method of Marmur (1961) (Appendix A.1), was digested with restriction endonucleases and separated by electrophoresis in Tris-acetate-buffered 1.2% agarose gels (Appendices A.1 and A.2). The DNA was transferred to GeneScreen membranes (New England Nuclear Corp., Boston, MA, USA) by the method developed by Smith and Summers (1980). Radioactively labelled plasmid pVS100 was used as a DNA probe and was prepared by nick translation with [α-^{32}P]dCTP as described by Rigby *et al.*,(1977) (Appendix A.1).

2.2.6. Studies on the capsule produced by transformed and untransformed *E. coli* JA221 cells

Single colonies of *E. coli* JA221 containing plasmid pVS100 were removed from sucrose MM plates, resuspended in sterile saline, negatively stained with india ink and viewed under a Zeiss photomicroscope using phase contrast illumination.

E. coli JA221 WT and JA221 transformed with plasmid pVS100 (Appendix A.1) were cultured separately or together on MM plates containing 6 mM sucrose or glucose(10 mM) and fructose(10 mM). *E. coli* YMC11 (pRM210) and LK111 (pVP105), used as controls, were propagated on MM plates supplemented with 10 mM glucose and 10 mM fructose.

2.2.7. DNA-directed cell-free protein synthesis

The synthesis of proteins by plasmid pVS100 was investigated in an *E. coli* DNA-directed cell-free system (Amersham, Procaryotic DNA-directed translation kit, Code N. 380). The procedure followed was as specified by the manufacturers, but quarter quantities were used throughout. The polypeptides were labelled with L-[^{35}S]methionine (specific activity 1040 Ci/mmol), and the incorporation of radioactivity was determined by liquid scintillation counting using 5 ml toluene-based scintillant and a Packard Tri-Carb 460 scintillation spectrometer. Sample volumes, containing 1-2 x 10^5 cpm of incorporated radioactivity were resolved by SDS-PAGE (Laemmli, 1970; O'Farrell, 1975) as described in Appendix A.1. The *in vitro* translated polypeptides were visualized by autoradiography.

2.2.8. Sucrase assays

Sucrase activities were assayed in sonicated cell-extracts and cell-free supernatant fluids unless otherwise stated. Cultures (100 ml) of *V. alginolyticus* and *E. coli* JA221 (pVS100) were grown overnight in MM (Appendix A.2) at 30 and 37^{o}C, respectively. Cells of a 10 ml sample were harvested by centrifugation at 12 000 x g for 10 min at 4^{o}C, the cell-free supernatant was kept on ice and the cells were washed in an equal volume of saline. The pellet was resuspended in 2 ml 0.05 M potassium phosphate buffer (pH 7.0) and sonicated in a MSE Soniprep 150 for three 30-s bursts at 4^{o}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 retained for enzyme assays.

Sucrase was determined by incubating 400 µl of 0.88 M sucrose in 0.05 M potassium phosphate buffer (pH 7.0) with 1 ml of the appropriate enzyme dilution at 37°C for 30 min. The enzyme reaction was terminated by adding a 1 ml sample to 3 ml dinitrosalicylic acid reagent (DNS) (Miller, 1959) (see Appendix A.2) and boiling the mixture for 5 min. The amount of reducing sugar liberated was determined spectrophotometrically at 510 nm. OD_{510} values were converted to µmoles of reducing sugar produced per ml per min using a standard curve prepared by plotting the absorbance (510 nm) against known concentrations of glucose solutions. Sucrase activities were determined in duplicate throughout and blanks, which were terminated at t=0 by the addition of DNS, were prepared for each sample.

In order to establish the reaction time corresponding to the linear portion of the enzymatic reaction curve, cell extracts were prepared from stationary phase *V. alginolyticus* and *E. coli* JA221 (pVS100) cultures. The volumes given for the sucrase enzyme assay were scaled up proportionally, to allow sampling from a single enzyme reaction at regular time intervals over 90 min. The optimum temperature for the sucrase activity assay was determined by incubating the reaction mixtures at 30, 37, 42 and 50°C for 30 min. The pH optimum was determined by diluting the preparations 56-fold with 0.05 M potassium phosphate buffers containing 0.88 M sucrose and adjusted to various pH values

(5.8, 6.0, 6.5, 7.1, 7.6, and 7.8). To determine whether the addition of NaCl would enhance the enzyme reaction, 54-fold dilutions of the cell extracts were prepared in potassium phosphate buffer containing 0.88 M sucrose. NaCl was added at various concentrations (0.0, 0.09, 0.4, 1.2 and 2.6 M) and the sucrase activity assayed. The stability of the sucrase enzyme was determined by storing the cell extracts at +4, -20 and -70°C and assaying a 50-fold dilution after 1,2,3, and 7 days.

2.3. RESULTS AND DISCUSSION

2.3.1. Cloning of the *V. alginolyticus* sucrose utilization system in *E. coli*

Transformation of *E. coli* JA221 with pEcoR251 containing *V. alginolyticus* chromosomal DNA fragments resulted in the isolation of six colonies which were able to utilize sucrose as a sole carbon source. Plasmids isolated from these colonies all retransformed *E. coli* JA221, and colonies able to utilize sucrose were obtained at the same frequency as Ap^r transformants. One plasmid was chosen for further studies and designated pVS100. Propagation of the *E. coli* transformant on MacConkey agar containing sucrose resulted in red colonies, which indicated the production of acid following sucrose hydrolysis.

2.3.2. Restriction endonuclease mapping and DNA hybridization

The restriction endonuclease map of pVS100 was obtained by complete single, double and triple digestions with a variety of restriction endonucleases (Fig. 2.1). The origin of the 10.4 kb insert in pVS100 was determined by Southern blotting and DNA hybridization with ^{32}P-labelled pVS100 (Fig. 2.2). Labelled pEcoR251 did not hybridize to *V. alginolyticus* chromosomal DNA (data not shown). *V. alginolyticus* total cellular DNA was digested with *Eco*RI, *Hin*dIII, *Bgl*I and *Sal*I restriction endonucleases and probed with ^{32}P-labelled pVS100. From the restriction map, hybridization of *V. alginolyticus* chromosomal DNA digested with *Eco*RI and *Bgl*I endonucleases would be expected to yield single bands on the autoradiogram, but two bands were observed (Fig. 2.2). Similarly, hybridization of *V. alginolyticus* chromosomal DNA digested with *Hin*dIII and *Sal*I would be expected to yield two bands, but three bands were observed. This suggests that either a region within the cloned 10.4 kb fragment might be duplicated in the *V. alginolyticus* genome or that the *V. alginolyticus* DNA was only partially digested in the four experiments with the different restriction enzymes. Control experiments with plasmids and phages indicated that the enzymes were all active and that they totally digested the control DNA. Gene amplification has been demonstrated in *V. cholerae* (Goldberg and Mekalanos, 1986). Alternatively, small repetitive extragenic sequences such as those reported for *E. coli* and *S. typhimurium* (Stern *et al.*, 1984) may be present in *V. alginolyticus*.

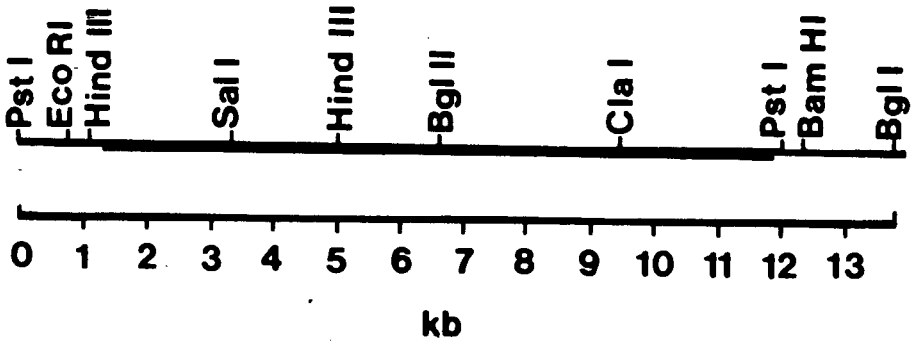

FIG. 2.1. Partial restriction endonuclease map of pVS100. Bold and thin lines represent *V. alginolyticus* and plasmid vector DNA respectively.

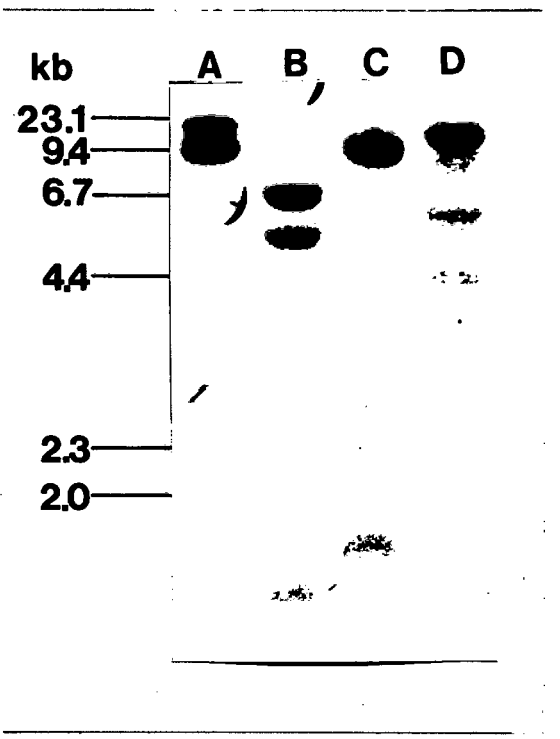

FIG. 2.2. Hybridization of ^{32}P-labelled pVS100 DNA with *V. alginolyticus* cellular DNA. Lanes A to D show an autoradiogram of various restriction endonuclease digestion products of *V. alginolyticus* cellular DNA after electrophoresis and transfer to a membrane hybridized with ^{32}P-labelled pVS100: A, *Eco*RI; B, *Hin*dIII C, *Bgl*I; D, *Sal*I.

2.3.3. Studies on the capsule produced by transformed and untransformed *E. coli* JA221 cells

E. coli JA221 (pVS100) colonies propagated on MM plates had an uncharacteristic mucoid appearance, a phenomenon usually attributed to the presence of capsular material. The capsule was shown not to consist of levan (possibly generated by a plasmid encoded levansucrase) by a method developed by Hestrin *et al.*, (1943). This was expected since previous inhibition studies with parahydroxymercuribenzoate indicated that the sucrose hydrolyzing activity encoded by plasmid pVS100 was mediated by a sucrase as opposed to a levansucrase (G. Blatch, personal communication). Parahydroxymercuribenzoate is an enzyme inhibitor used to distinguish between these two enzymes. Sucrase is completely inhibited by parahydroxymercuribenzoate whereas levansucrase is resistant under similar conditions (Lepesant *et al.*, 1976).

The mucoid colony morphology occurred when *E. coli* JA221 transformants were propagated on MM plates supplemented with either sucrose or glucose and fructose. Furthermore, untransformed *E. coli* JA221 displayed the same colony morphology when propagated on MM medium. The presence of a capsule was confirmed by viewing negatively stained cells under a microscope using phase contrast illumination (Fig.2.3). Colonies of two randomly chosen *E. coli* transformants, YMC11 (pRM210) and LK111 (pVP105), did not have a mucoid appearance when propagated on MM plates containing glucose and fructose. This confirmed that the mucoid morphology was an inherent property of *E. coli*

JA221 colonies grown on MM media and was independent of the presence of pVS100.

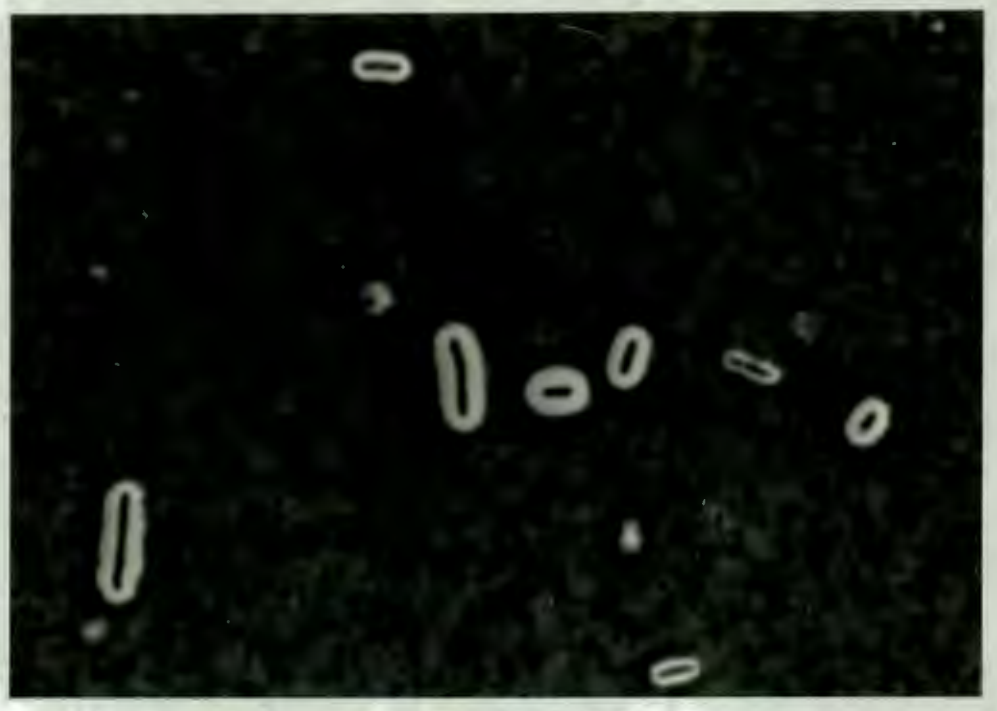

FIG. 2.3. Phase contrast photomicrograph of *E. coli* JA221 (pVS100) propagated on sucrose MM plates. Cells were negatively stained with india ink (mag. 10 x 100).

2.3.4. DNA-directed cell-free protein synthesis

Cell-free coupled transcription-translation of pVS100 resulted in the production of three major proteins with apparent M_r of approximately 58 000, 50 000 and 32 000 (Fig. 2.4). The polypeptide of apparent M_r of approximately 31 000 produced by pEcoR251 and pVS100 was the β-lactamase gene product (Sancar *et al.*, 1979). The additional polypeptide products detectable in the pEcoR251 control sample were associated with the *Eco*RI gene and were not detectable in pVS100 since cloning into pEcoR251 inactivated the *Eco*RI gene. The reported M_r for other sucrose hydrolyzing enzymes

ranges between 28 000 and 110 000 (Lepesant *et al.*, 1976; Pascal *et al.*, 1971; Thompson and Chassy, 1981; Kunst *et al.*, 1974; Schmid *et al.*, 1982; Lunsford and Macrina, 1986).

FIG. 2.4. Autoradiogram of the cell-free coupled transcription-translation polyacrylamide gel. Lane A: [^{35}S]-labelled protein standards (in decreasing order of M_r): ovotransferrin (hen), ovalbumin (hen), lactate dehydrogenase (pig heart), carbonic anhydrase (bovine erythrocyte), myoglobin (equine). Lane B: Zero DNA control. Lane C and D: Translation products of *in vitro* transcribed and translated plasmids. Lane C: pEcoR251, Lane D: pVS100.

2.3.5. Optimization of sucrase enzyme activity assays

Sucrase activity assayed in *V. alginolyticus* and *E. coli* JA221 (pVS100) cell extracts displayed a linear response between 0 and 45 min, after which the curve flattened out. The temperature optimum for sucrase activity was 37°C. At temperatures of 30, 42 and 50°C, sucrase activity decreased by 31, 26 and 69% respectively. The pH optima for sucrase activity in cell extracts prepared from *V. alginolyticus* and JA221 (pVS100) cells was pH 7.1 and pH 7.6 respectively. The addition of NaCl caused a reduction of 6 to 35% in sucrase activity in cell extracts prepared from *V. alginolyticus*, whereas cell extracts prepared from the *E. coli* transformants showed a small increase in activity of 11% in 0.09 M NaCl. Cell extracts from both *V. alginolyticus* and JA221 (pVS100) retained their sucrase activity over 7 days when stored at either -20°C or -70°C. Storage at 4°C however resulted on average in a 60% loss of activity after each day.

All subsequent sucrase assays were therefore carried out for 30 min at 37°C and pH 7.0 without the addition of salt. Samples were stored routinely at -70°C for no longer than a week.

2.4. CONCLUSION

A 10.4 kb chromosomal DNA fragment from *V. alginolyticus*, containing the genes mediating sucrose metabolism, conferred a stable sucrose-positive phenotype

onto *E. coli* transformants. This was particularly encouraging, since cloning of sucrase enzymes from other bacteria into *E. coli* did not necessarily result in the generation of transformants able to utilize sucrose as a sole carbon source although expression of the heterologous gene was demonstrated (Fouet *et al.*, 1982).

E. coli contains no endogenous sucrase (Fouet *et al.*, 1982; 1986), but is often the organism of choice for carrying out industrial fermentations based on recombinant DNA techniques. Hence, the possibility of using plasmids like pVS100 as vectors in biotechnology processes is important since it would permit the utilization of cheap sucrose containing substrates in *E. coli* fermentations.

Preliminary studies have indicated that 1% low grade molasses supported the growth of *E. coli* JA221 (pVS100) (A. Hromic, personal communication).

Having established the optimal conditions for the sucrase assay, the groundwork was prepared for studies on the regulation and cellular distribution of the sucrase in *V. alginolyticus* and *E. coli*.

CHAPTER THREE

STUDIES ON THE *V. ALGINOLYTICUS* SUCRASE

CHAPTER THREE

STUDIES ON THE *V. ALGINOLYTICUS* SUCRASE

Summary: Sucrase activity was detected both intra- and extracellularly in *E. coli* JA221 (pVS100) cultures . In *V. alginolyticus* only intracellular sucrase was detected. Sucrase synthesis in *E. coli* JA221 (pVS100) was induced by sucrose and repressed by glucose and the repression was reversed by cAMP. The same responses were observed for sucrase synthesis in *V. alginolyticus*. Transformation of pVS100 into *E. coli* mutant strains deficient in the catabolite activator protein (CAP) and adenylate cyclase, confirmed that at least functional CAP is required for the induction of the sucrose utilization system. Localization of sucrase activity in *E. coli* JA221 (pVS100) by osmotic shock indicated that on average 76% of the sucrase activity was found in the cytoplasm while the remaining 24% was variably distributed between the periplasm and the extracellular supernatant fluid. The presence of sucrase in the supernatant was attributed to non-specific leakage across the periplasmic membrane. Results obtained from cytoplasmicly located control enzymes however, ruled out the possibility of protein leakage across the cytoplasmic membrane. Experiments with Tn*phoA* indicated that translocation of sucrase across the cytoplasmic membrane was mediated by a mechanism which did not involve a signal sequence.

3.1. INTRODUCTION

The literature describing the characterization of sucrase enzymes synthesized by gram-positive and gram-negative bacteria has been discussed in section 1.2 of the General Introduction. The sucrase enzyme from *V. alginolyticus* differs in one aspect from all other sucrases studied, in that its primary substrate is sucrose as opposed to sucrose-6-phosphate (Kakinuma and Unemoto, 1985). This is due to the fact that sucrose translocation across the *V. alginolyticus* membrane proceeds by a mechanism not involving concomitant phosphorylation.

The studies done on the regulation and cellular distribution of the *V. alginolyticus* sucrase in both the wild type *V. alginolyticus* and the recombinant *E. coli* are discussed in this chapter.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains, plasmids and phages

Sucrase activity assays were performed in *E. coli* JA221 (pVS100) and *V. alginolyticus* (Section 2.2.1). *E. coli* mutants CA8445 (*cya*$^-$ *crp*$^-$ Str^r), CA8445.1 (HfrH *crp*$^-$ Str^r) and the parental strain CA8000 were kindly supplied by Jon Beckwith and were used to study CAP-dependence during catabolite repression. The PhoA$^-$ *E. coli* strain CC118 [*ara D*139 Δ(ara, leu)7697 Δ*lacX*74 *phoA*Δ20 *galE galK* thi *rpsE rpoB* $argE_{am}$ *recA*1] (Manoil and Beckwith, 1985) was used as the host cell for the Tn*phoA* fusion experiment. Phage λ:Tn*phoA* *b*221 *cI*857 P_{am}3 with Tn*phoA* in or near *rex* was a gift from C. Manoil (Gutierrez *et al.*, 1987). Plasmid pVS106, containing the sucrose utilization system subcloned from pVS100 into vector pBR325 (Bolivar, 1978), was constructed in this study. Plasmid pVS100 was described in Chapter 2.

3.2.2. Standard methods, buffers and media

Appendix A lists standard techniques, buffers and media.

3.2.3. Regulatory studies

Cultures of *V. alginolyticus* and *E. coli* JA221 (pVS100) were grown overnight in either MM or LB broth at 30 and 37^oC, respectively. MM was supplemented with sucrose (6 mM) or glucose (10 mM). The overnight cultures were diluted 10-fold into 200 ml medium. Whenever the composition of the medium used for the overnight culture did not correspond to

that of the main culture, the cells were washed in an equal volume 1 x CSH MM salts (Appendix A.2) (7700 x g for 10 min at room temperature) before inoculating the main culture. Cultures were sampled at regular time intervals until early stationary phase and the samples (10 ml) were processed as described in Section 2.2.8. Sucrase activity was assayed in sonicated cell extracts and cell-free supernatants (Section 2.2.8). The effect of glucose and cAMP on sucrase activity in *V. alginolyticus* and *E. coli* JA221 (pVS100) was studied by adding 5 mM cAMP and/or 10 mM glucose to mid-exponential-phase cultures growing in 6 mM sucrose MM.

3.2.4. Studies on catabolite repression using *E. coli crp*$^-$ and *cya*$^-$ mutants

Plasmid pVS100 was transformed into the following competent *E. coli* strains: JA221, CA8000, CA8445 and CA8445.1 (Appendix A.1). Transformants were spread onto LB or sucrose MM agar plates containing Str (150 μg/ml) and/or Ap (50 μg/ml) (Appendix A.2).

3.2.5. Studies on the cellular localization of the sucrase activity in *E. coli* JA221 (pVS100) cells

Periplasmic and cytoplasmic cell fractions were prepared by the osmotic-shock method as described by Willis *et al.*, (1974). Cultures (100 ml) of *E. coli* JA221 (pVS100) were grown to mid-exponential phase (OD_{600}=0.6) in sucrose MM supplemented with 10 mM IPTG at 37^{o}C. Cells were harvested by centrifugation at 8000 x g for 6 min at 4^{o}C. The supernatant fluid was retained and the cells washed in an equal volume of 1 x CSH MM salts (Appendix A.2). This

was followed by a second wash in 100 ml 1 x CSH MM salts containing 30 mM NaCl and 30 mM Tris-HCl pH 7.3. The pellet was resuspended in 10 ml/g wet weight of 30 mM Tris-HCl pH 7.3 and an equal volume of TSE (30 mM Tris-HCl, pH 7.3, 40% sucrose and 2 mM Na EDTA). The cell suspension was centrifuged at 8000 x g at 4°C for 6 min and the supernatant retained as the primary periplasmic cell fraction. Within 1 min, one supernatant-volume of cold, sterile, deionized water and 1 mM $MgCl_2$ (final concentration) was added to the cells, which were resuspended gently and subsequently harvested at 8000 x g for 6 min at 4°C. The supernatant was retained as the secondary periplasmic cell fraction, and the pellet was rinsed gently with 0.05 M potassium phosphate buffer pH 7.0 before being resuspended in 5 ml of the same buffer. The 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 retained as the cytoplasmic fraction. Sucrase, β-lactamase and β-galactosidase were assayed in the cell-free culture supernatants, the cytoplasmic and primary and secondary periplasmic cell fractions.

3.2.6. Enzyme assays

The methods developed by Sykes and Nordstrom (1972) and Pardee *et al.*, (1959) were utilized to assay for β-lactamase and β-galactosidase enzyme activities respectively. To assay β-lactamase activity, 1 ml of starch-iodine solution (Appendix A.2) was added to 1 ml of substrate solution and

made up to 2.9 ml with potassium phosphate buffer (0.1 M, pH 5.9). Substrate solutions were freshly prepared daily by dissolving 74 μg of the sodium salt of Ap per ml potassium phosphate buffer (0.1 M, pH 5.9) (final concentration 0.2 mM) and kept on ice for the period of the experiment. The mixture was incubated at 30^{o}C for 5 min before the reaction was initiated by the addition of 0.1 ml of appropriately diluted cell fractions. Dilutions were prepared in 0.1 M potassium phosphate buffer (pH 5.9). The reaction was allowed to continue for 20 min at 30^{o}C before being terminated by the addition of 1 ml of ice cold 1% (w/v) trichloroacetic acid. The absorbance was read immediately at 620 nm. All enzyme assays were performed in duplicate and a blank was prepared for each sample by omitting the addition of the substrate solution.

The activity of β-galactosidase was assayed by incubating a 0.5 ml sample of the appropriately diluted cell fraction at 28^{o}C for 5 min before adding 100 μl of ONPG (13 mM in 250 mM potassium phosphate buffer pH 7.0). Dilutions were prepared in 50 mM potassium phosphate buffer pH 7.0. The reaction was terminated after 6 min by the addition of 250 μl of 14% (w/v) Na_2CO_3 and 3 ml water, and the optical density was measured at 430 nm. All enzyme assays were performed in duplicate and a reaction blank was prepared by substituting the cell fraction-sample with 0.5 ml 50 mM potassium phosphate buffer pH 7.0.

Sucrase activities were assayed in duplicate as outlined in Section 2.2.8 and blanks were prepared for each reaction.

The enzyme activities in the three cell fractions were expressed as a percentage of the total activity regarding the activity in the periplasm to be the sum of the primary and secondary periplasmic cell fraction activities.

3.2.7. λ:Tn*phoA* mutagenesis

3.2.7.1. Construction of pVS106

The 2.8 kb *Cla*I-*Pst*I fragment of pVS100 was subcloned into *Cla*I-*Bam*HI endonuclease digested pBR325, inactivating the Tet^r gene carried on this vector. Prior to the endonuclease digestions with *Cla*I, the sticky ends generated by *Bam*HI and *Pst*I endonucleases had to be filled in to allow a blunt end ligation of pBR325 to the pVS100 fragment. Restriction fragments to be filled in were purified by two phenol and two ether extractions followed by an ethanol precipitation in the presence of 0.3 M sodium acetate. Filling-in reactions were carried out in PolI buffer (Appendix A.2) in the presence of 1u/μg DNA of Klenow enzyme (Boehringer Mannheim) and 250 μM of each dNTP. Reactions were allowed to proceed for 10 min at 37°C before being terminated by another purification as outlined above. The resulting plasmid was designated pVS106. The ligation reaction and transformation of pVS106 into the $PhoA^-$ *E. coli* strain CC118 was carried out as described in Appendix A.1. The transformants were propagated on LB agar containing Clm (40 μg/ml).

3.2.7.2. λ:Tn*phoA* mutagenesis

Transposon insertions into pVS106 were obtained by a modification of the method developed by de Bruijn and Lupski (1984). A 5 ml culture of *E. coli* CC118 (pVS106) was grown overnight at 30°C in LB broth containing Clm (40 μg/ml) and 10 mM $MgSO_4.7H_2O$. Phage λ:Tn*phoA* was added at a multiplicity of infection of approximately 1.0 to 1 ml of *E. coli* CC118 (pVS106) cells and was incubated for 15 min at 30°C. Aliquots of 0.1 ml of the mixture were subsequently diluted 10-fold with LB broth and incubated for a further 3 h at 30°C ensuring good aeration. The cells were concentrated by brief centrifugation in an Eppendorf microfuge and transposon insertions were selected on LB agar supplemented with Clm (40 μg/ml), Kan (250 μg/ml and 5-bromo-4-chloro-3-indolyl phosphate (80 μg/ml). After incubation at 30°C for 2-3 days, plasmid DNA prepared from blue, alkaline phosphatase-positive colonies was restriction endonuclease mapped (Appendix A.1).

3.2.7.3. DNA sequence determination

A 15 nt synthetic oligonucleotide primer (5' AAACGGCGAGCACCG 3'), was used to provide DNA sequence information across the junction of the fusions between the alkaline phosphatase gene and pVS106 (Appendix A.1). The primer was complementary to the DNA sequence corresponding to positions +126 to +140 of the *phoA* gene.

3.3. RESULTS AND DISCUSSION

3.3.1. Regulatory studies

Supernatant and intracellular sucrase activities were assayed throughout the growth cycle of *V. alginolyticus* and *E. coli* JA221 (pVS100) (Fig. 3.1). Both bacteria synthesized intracellular sucrase activity throughout the growth cycle in sucrose MM. No extracellular sucrase activity was detected in the *V. alginolyticus* cultures, whereas the *E. coli* JA221 (pVS100) transformant produced extracellular sucrase activity during growth. The levels of intracellular sucrase in *E. coli* containing pVS100 were approximately fivefold higher than in the *V. alginolyticus* strain. This could have been due to an effect of the gene copy number.

Expression of sucrase activity in *E. coli* JA221 (pVS100) required the presence of sucrose as an inducer (Fig. 3.2 B), and no sucrase activity was detected in glucose MM. The same requirement for sucrose as an inducer was observed when *E. coli* JA221 (pVS100) cells were propagated in LB medium. Sucrase activity was only detected when the LB broth was supplemented with 6 mM sucrose (Fig. 3.3). The cloned DNA fragment from *V. alginolyticus* may contain a regulatory gene whose action allowed sucrose to activate sucrase production. It is also possible that *E. coli* JA221 contained an analogous fortuitous repressor protein which recognized the cloned DNA fragment and was able to complex with sucrose.

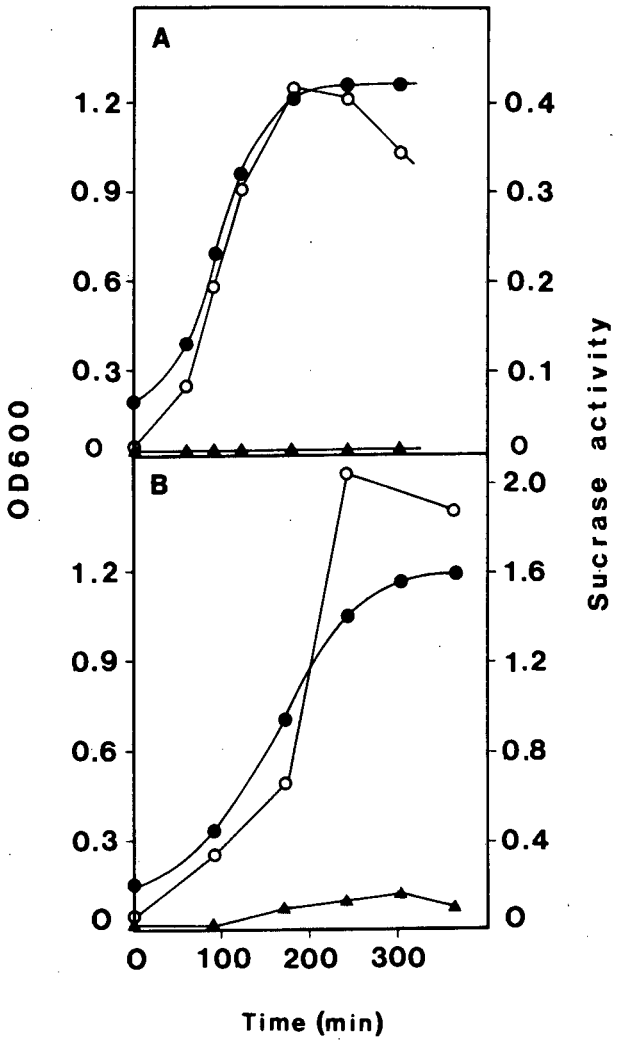

FIG. 3.1. Sucrase activities as a function of growth in *V. alginolyticus* (A) and *E. coli* JA221 (pVS100) (B). Sucrase activities are expressed as μmoles of reducing sugar produced per ml per min. Symbols: ●, cell growth curve, (optical density at 600 nm [OD_{600}]); O, intracellular sucrase; ▲, extracellular sucrase.

The effect of glucose on sucrase activity in *E. coli* JA221 (pVS100) was determined (Fig. 3.2 B). Sucrase synthesis in mid-exponential-phase cultures growing in 6 mM sucrose MM was inhibited by the addition of 10 mM glucose. This glucose-induced repression of sucrase synthesis in mid-exponential-phase *E. coli* JA221 (pVS100) cells was reversed by the simultaneous addition of 5 mM cAMP. In *V. alginolyticus*, sucrase synthesis was also induced by

sucrose and repressed by glucose, and the repression was reversed by cAMP (Fig. 3.2 A). This suggested, that the cloned *V. alginolyticus* fragment presumably contained a CAP binding site. Since no genes on the vector pEcoR251 were regulated by sucrose, it was concluded that sucrase production in *E. coli* was expressed from a *V. alginolyticus* regulatory region on pVS100.

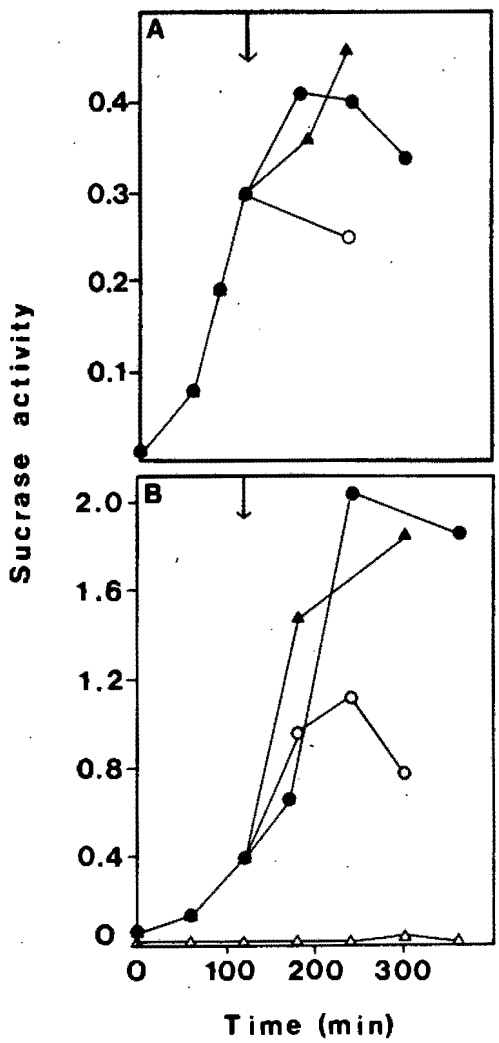

FIG. 3.2. Induction and repression of intracellular sucrase synthesis in *V. alginolyticus* (A) and *E. coli* JA221 (pVS100) (B) by sucrose and glucose, respectively. An *E. coli* JA221 (pVS100) culture grown in glucose MM was resuspended in glucose MM (Δ). Cultures grown in sucrose MM were resuspended in sucrose MM (●). After 120 min (arrow), 10 mM glucose (O) or 10 mM glucose-5 mM cAMP (▲) was added. Sucrase activities of the cultures are expressed as μmoles of reducing sugar produced per ml per min.

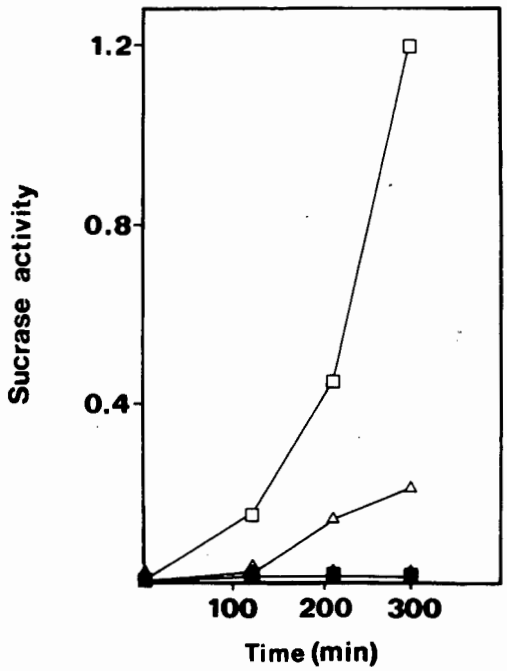

FIG. 3.3. Induction of intra- and extracellular sucrase synthesis in *E. coli* JA221 (pVS100) by sucrose. Intra- and extracellular sucrase activities of cultures grown in LB broth are indicated by (■) and (▲), respectively. Intra- and extracellular sucrase activities of cultures grown in LB broth containing 6 mM sucrose are indicated by (□) and (△), respectively. Sucrase activities are expressed as μmoles of reducing sugar produced per ml per min.

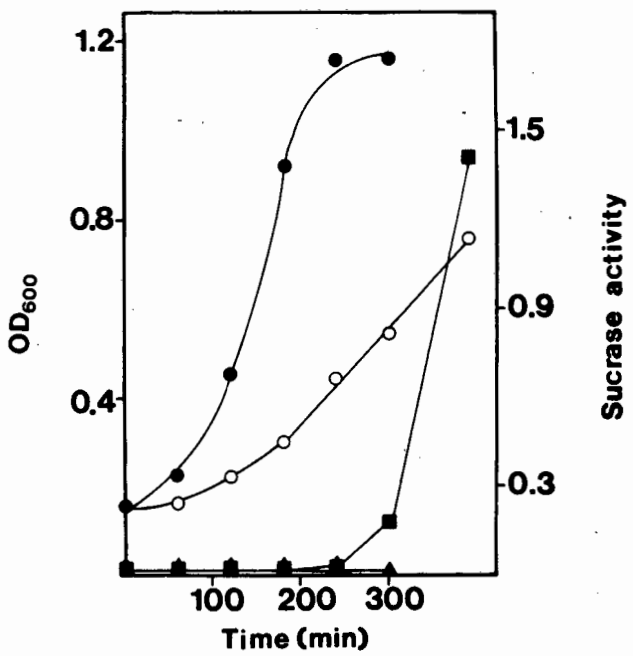

FIG. 3.4. Time of intracellular sucrase induction in uninduced *E. coli* JA221 (pVS100) cells. Cultures grown in glucose MM were resuspended in glucose MM (▲) and sucrose MM (■). Cell growth curves (optical density at 600 nm [OD_{600}]) in glucose and sucrose MM are indicated by (●) and (○), respectively. Sucrase activities are expressed as μmoles of reducing sugar produced per ml per min.

The time required for sucrase induction in *E. coli* JA221 (pVS100) cells was determined by inoculating uninduced cells (propagated on glucose MM) into sucrose MM and assaying for supernatant and intracellular sucrase activities throughout the growth cycle. The rate of growth of uninduced cells was significantly reduced when compared to that of fully induced *E. coli* JA221 transformants (Figs. 3.1 and 3.4). After 4 h of incubation, which corresponded to the early exponential phase of the culture, sucrase synthesis started.

3.3.2. Studies on catabolite repression using *E. coli crp*$^-$ and *cya*$^-$ mutants

E. coli mutants CA8445.1 (mutation in the *crp* gene encoding CAP) and *E. coli* CA8445 [mutation in both the *crp* gene and the *cya* gene (coding for adenylate cyclase)] were transformed with plasmid pVS100. Transformants of the control strain JA221 and the parental strain CA8000 were able to metabolize sucrose as the sole source of carbon when propagated on sucrose MM, giving rise to colonies with the characteristic mucoid morphology (Chapter 2). *E. coli* strains CA8445 (pVS100) and CA8445.1 (pVS100) however, were unable to grow on sucrose MM plates. This suggested that at least a functional CAP and possibly both a functional CAP and adenylate cyclase, were required for induction of the sucrose utilization system permitting growth on sucrose MM.

3.3.3. Studies on the cellular localization of the sucrase activity in *E. coli* JA221 (pVS100) cells

The localization of the sucrase activity of *E. coli* JA221 (pVS100) was determined in cell fractions prepared by osmotic shock and the results are summarized in Table 3.1. The localization of β-galactosidase and β-lactamase, as control cytoplasmic and periplasmic enzymes, respectively, was determined in the same cultures. The majority of the β-galactosidase activity was detected in the cytoplasm, whereas the majority of the β-lactamase activity was present in the periplasm. The majority of the sucrase activity was in the cytoplasm, and the remainder of the sucrase activity (average 24%) appeared to be variably distributed between the periplasm and extracellular supernatant fluid. Up to approximately 20% of the sucrase activity in *E. coli* JA221 (pVS100) cultures was detected in the extracellular supernatant fluid.

TABLE 3.1. Localization of the sucrase activity in *E. coli* JA221 (pVS100) cells

Enzyme	Enzyme activity [a]		
	Cytoplasm	Periplasm	Extracellular supernatant fluid
β-Lactamase	12.5-15.3	55.0-62.6	24.6-29.7
β-Galactosidase	90.8-94.4	2.4-3.5	3.2-6.1
Sucrase	67.7-84.9	2.3-20.8	9.2-21.5

[a] Enzyme activities are expressed as a percentage of the total enzyme activity. Experiments were performed in triplicate, and the range of values is given.

3.3.4. λ:Tn*phoA* mutagenesis

E. coli JA221 cells containing pVS106 were shown to be sucrase-positive by assaying cell extracts as outlined in section 2.2.8. Tn*phoA* mutagenesis carried out on pVS106 resulted in the isolation of several recombinant plasmids conferring alkaline phosphatase activity on the PhoA$^-$ recipient *E. coli* strain CC118. Since the alkaline phosphatase moiety of Tn*phoA* does not contain a signal sequence (Manoil and Beckwith, 1985; Von Heijne, 1983), it requires an in-frame sequence upstream from the transposon insertion site to function as a signal sequence for export and alkaline phosphatase activity. DNA sequencing by the chain termination method, employing a synthetic primer complementary to the *phoA* gene, was used to determine the exact point of insertion of the transposon. For all alkaline phosphatase positive recombinant plasmids isolated, the point of insertion was identified to be downstream and in frame with the β-lactamase signal sequence contained on vector pBR325. Despite repeated attempts, no Tn*phoA* positive insertions were detected within the *V. alginolyticus* derived DNA insert. It was interesting to note, that the insertion of the 7.6 kb Tn*phoA* transposon into the N-terminal region of the β-lactamase gene carried on plasmid pBR325 did not disrupt or reduce the β-lactamase activity.

Control experiments carried out in parallel, utilizing the *V. alginolyticus* exoprotease A gene cloned into pBR325 resulted in the isolation of 6 recombinant plasmids with

transposons inserted into the exoprotease A gene. This led to the identification of a classical signal sequence at the N-terminus of the cloned exoprotease A gene (S. Deane, personal communication). The inability to obtain positive Tn*phoA* insertions within the *V. alginolyticus* derived DNA insert in pVS106, whereas positive insertions were readily obtained in the β-lactamase and exoprotease A genes, suggested that the *V. alginolyticus* sucrase in *E. coli* is not exported by means of a signal sequence.

Studies on the cellular localization of the sucrase activity in *E. coli* JA221 (pVS100) cells (Section 3.3.3), indicated that there was no leakage of β-galactosidase from the cytoplasm. However, 24-29% of β-lactamase leaked from the periplasm into the supernatant fluid. This indicated that the translocation of the *V. alginolyticus* sucrase from the cytoplasm into the periplasm in *E. coli* could not be accounted for by non-specific leakage. Translocation of the sucrase across the *E. coli* cytoplasmic membrane however, was mediated by some mechanism which did not involve a signal sequence.

3.4 CONCLUSION

The 10.4 kb chromosomal fragment from *V. alginolyticus* contained on plasmid pVS100, encoded a sucrase enzyme which was inducible and its synthesis was shown to be under catabolite regulation. In contrast to *V. alginolyticus*, sucrase activity was detected both intra- and extracellularly in *E. coli* JA221 cells harbouring pVS100.

Studies on the cellular localization of the sucrase activity in *E. coli* JA221 (pVS100) cells indicated that sucrase was primarily a cytoplasmic enzyme translocated by a mechanism not involving a signal sequence across the cytoplasmic membrane into the periplasm. Translocation of sucrase from the periplasm into the supernatant fluid appeared to proceed by non-specific leakage through the periplasmic membrane. The mechanism of translocation of the *V. alginolyticus* sucrase across the *E. coli* cytoplasmic membrane is not known and remains to be elucidated.

CHAPTER FOUR

STUDIES ON THE *V. ALGINOLYTICUS* SUCROSE UPTAKE SYSTEM

CHAPTER FOUR

STUDIES ON THE *V. ALGINOLYTICUS* SUCROSE UPTAKE SYSTEM

Summary: *V. alginolyticus* and *E. coli* JA221 (pVS100) cells were shown to actively transport sucrose by an inducible, Na^+-independent sucrose transport system. Sucrose was translocated both in the presence and absence of Na^+, K^+ and Li^+. The maximum velocity and apparent K_m values of sucrose uptake for the *V. alginolyticus* strain and *E. coli* JA221 (pVS100) were 130 nmol/mg of protein per min and 50 μM and 6 nmol/mg of protein per min and 275 μM, respectively.

4.1. INTRODUCTION

Several sucrose transport systems from gram-negative and gram-positive organisms are discussed in section 1.4 of the General Introduction. With the exception of *V. alginolyticus* and several other marine bacteria, the transport of sucrose in all other organisms was reported to be mediated by a PEP-dependent PTS resulting in the intracellular accumulation of phosphorylated sucrose. *V. alginolyticus*, on the other hand, translocates sucrose by means of a Na^+ electrochemical gradient established by a Na^+/H^+ antiport or by a primary Na^+ pump (Tokuda and Unemoto, 1983; Kakinuma and Unemoto, 1985).

It appeared likely that plasmid pVS100 contained the respective gene(s) encoding the *V. alginolyticus* sucrose

transport system for three reasons. Firstly, the *V. alginolyticus* derived insert in plasmid pVS100 is very large (10.4 kb). Secondly, pVS100 encoded three polypeptides and finally, the fact that most reported genes, encoding transport functions, are closely linked on the chromosome to the gene(s) for the respective hydrolytic enzyme(s).

Studies done on the *V. alginolyticus* sucrose transport system in both the wild type *V. alginolyticus* and the recombinant *E. coli* are discussed in this chapter.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains and plasmids

V. alginolyticus, *E. coli* JA221 and plasmid pVS100 have been described in Section 2.2.1.

4.2.2. Standard methods, buffers and media

Appendix A lists standard techniques, buffers and media.

4.2.3. Processing of membrane filters and liquid scintillation counting

Samples of 50 µl were withdrawn from the uptake reaction mixture at regular time intervals and filtered through membrane filters (type HA, pore size 0.45 µm; Millipore Corp.). Membrane filters were soaked in sterile, distilled water for 14 h before use, and rinsed with 2 ml washing solution after filtration. After being dried, the filters were counted in 5 ml toluene-based scintillant in a Packard Tri-Carb 460 scintillation spectrometer. To determine the total counts present in each uptake reaction mixture, an unfiltered 50 µl sample was counted in the presence of 5 ml scintillant.

4.2.4. Sugar competition studies on the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

Cultures (20 ml) of *V. alginolyticus*, *E. coli* JA221 and *E. coli* JA221 (pVS100) were grown overnight at 30°C (*V. alginolyticus*) and 37°C (*E. coli*) in sucrose MM

(*V. alginolyticus* and *E. coli* JA221 (pVS100) and glucose MM (*E. coli* JA221) (Appendix A.2). The cells were harvested by centrifugation at 6000 x g for 6 min at room temperature, washed twice in 20 ml 1 x CSH MM salts and resuspended in an equal volume of 1 x CSH MM salts. A 1 ml sample of each cell suspension was retained for the determination of the protein concentration as outlined in Section 4.2.8. The cell suspensions were kept at room temperature throughout the experiment.

Uptake reactions were started by mixing 0.5 mM [^{14}C] sucrose (specific activity 10 Ci/mol, Amersham) (final concentration) with 400 μl of the cell suspension. The effect of other sugars on the uptake of radioactively labelled sucrose was determined by adding sucrose, glucose or fructose at 15, 28 and 28 mM final concentrations, respectively, to the uptake reaction mixture at time zero. Samples (50 μl) were withdrawn and filtered at regular time intervals over 20 min, rinsed with 2 ml of 1 x CSH MM salts and processed as described in Section 4.2.3. The uptake of [^{14}C]sucrose was expressed as μmoles per mg of protein.

4.2.5. Studies on the salt dependence of [^{14}C]sucrose uptake by *V. alginolyticus* and *E. coli* JA221 (pVS100)

Late-exponential-phase cultures of *V. alginolyticus* and *E. coli* JA221 (pVS100) were grown in Tokuda sucrose MM (Tokuda *et al.*, 1981; Appendix A.2) at 30 and 37^{o}C, respectively. *V. alginolyticus* cells (20 ml) were harvested by centrifugation at 6000 x g for 6 min at 4^{o}C and washed in 300 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4

and 50 mM Tris-HCl (pH 7.2) (MacLeod and MacLeod, 1986). The pellet was resuspended in 4 ml of the same solution and the suspension was kept on ice. *E. coli* JA221 (pVS100) cells (21 ml) were harvested by centrifugation at 6000 x g for 6 min at room temperature and washed twice with an equal volume of 50 mM diethanolamine HCl-0.4 M CsCl (pH 8.0) (Tokuda *et al.*, 1982 a), holding the cells for 10 min at room temperature between washes. The cells were washed two more times with 21 ml 0.05 M potassium phosphate buffer (pH 6.9) containing 0.1 mM $MgSO_4$ and were concentrated in 2 ml of the same solution. The suspension was kept at room temperature throughout the experiment. Samples of both the *V. alginolyticus* and the *E. coli* JA221 (pVS100) cell suspensions were retained for protein concentration determinations.

V. alginolyticus cells (25 μl) were added to 365 μl incubation buffer, which was identical to the choline chloride wash solution. In experiments, studying the effect of salts other than choline chloride on the uptake of [^{14}C]sucrose, choline chloride was replaced by 0.3 M NaCl, KCl or LiCl. *E. coli* JA221 (pVS100) cells (50 μl) were added to 330 μl of incubation buffer containing 0.1 mM $MgSO_4$ and 0.1 M NaCl, KCl or LiCl in 0.05 M potassium phosphate buffer (pH 6.9). The uptake of [^{14}C]sucrose in *E. coli* JA221 transformants was determined in the presence of 12.5 mM glucose and 12.5 mM fructose.

After an adaptation period of 30 s, all uptake experiments were started by the addition of 250 μM [^{14}C]sucrose (specific activity 10 Ci/mol, Amersham) (final

concentration). Samples (50 μl) were withdrawn and filtered at regular time intervals over 4 min. The filters were rinsed with 2 ml of the respective incubation buffer and processed as outlined in Section 4.2.3. Methanol and the uncoupler CCCP (10 μM final concentration) dissolved in methanol were added 7 min prior to the addition of the radioactively labelled sucrose. The uptake of [^{14}C]sucrose was expressed as μmoles per mg of protein.

4.2.6. Kinetic studies on the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

Late-exponential-phase cultures of *V. alginolyticus* and *E. coli* JA221 (pVS100) were grown in sucrose MM containing 0.2% succinate and Tokuda sucrose MM (Appendix A.2), respectively. *V. alginolyticus* cells (20 ml) were harvested by centrifugation at 6000 x g for 6 min at room temperature, washed in an equal volume of 1 x CSH MM containing 0.2% succinate and were resuspended in 20 ml of the same solution supplemented with Clm (100 μg/ml). *E. coli* JA221 (pVS100) cells (20 ml) were harvested, washed in 10 ml 0.05 M potassium phosphate buffer containing 0.1 mM $MgSO_4$ and resuspended in an equal volume of the same solution. The cell suspensions were kept at room temperature throughout the experiment and samples were retained for the protein concentration determinations.

Uptake of radioactively labelled sucrose by *V. alginolyticus* cells was started by adding 390 μl of the cell suspension to [^{14}C]sucrose (specific activity 10 Ci/mol, Amersham) so as to obtain final concentrations of

sucrose ranging from 5 to 20 μM. [^{14}C]sucrose uptake by *E. coli* JA221 transformants was determined in the presence of a 50-fold molar excess of unlabelled glucose and fructose to [^{14}C]sucrose. After a 30 s adaptation period, uptake reactions were started by the addition of radioactively labelled sucrose to 370 μl of the *E. coli* cell suspension so as to obtain a final concentration of sucrose ranging from 50 to 350 μM. Samples of 50 μl were withdrawn from the uptake reactions and filtered at 10 s intervals over 1 min, and the membranes were rinsed with 2 ml of the respective washing solution used for the preparation of the cell suspension. The membranes were processed as outlined in Section 4.2.3. Uptake was determined within 20 s after the addition of [^{14}C]sucrose, and one nmole of [^{14}C]sucrose was represented by 20 000 cpm. The maximum velocities (V_{max}) and apparent K_m values of sucrose uptake in *V. alginolyticus* and *E. coli* JA221 (pVS100) were determined from Lineweaver-Burk plots after a least square regression analysis. The uptake velocities (V) and substrate concentrations (S) were expressed as nmole sucrose/mg of protein per min and μM, respectively.

4.2.7. Studies on the induction of the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

Late-exponential-phase cultures of *V. alginolyticus* and *E. coli* JA221 (pVS100) were grown in MM and Tokuda MM, respectively, each supplemented with either sucrose or glucose (Appendix A.2). *V. alginolyticus* cells (20 ml) were harvested by centrifugation at 6000 x g for 6 min at room

temperature, washed in an equal volume of 1 x CSH MM salts and resuspended in 20 ml of the same solution. *E. coli* JA221 (pVS100) cells (20 ml) were harvested, washed in 0.05 M potassium phosphate buffer (pH 6.9) containing 0.1 mM $MgSO_4$ and resuspended in an equal volume of the same solution. All cells were kept at room temperature throughout the experiment and samples of the cell suspensions were retained for protein concentration determinations.

Uptake of [^{14}C]sucrose (specific activity 10 Ci/mol, Amersham) by *V. alginolyticus* and *E. coli* JA221 transformants was started by the addition of 250 μM (final concentration) of labelled sucrose to 390 and 380 μl of cell suspensions, respectively. In *E. coli* JA221 (pVS100) cells, uptake of sucrose was determined in the presence of 15 mM glucose and 15 mM fructose after an adaptation period of 30 s. Samples of 50 μl were withdrawn and filtered at regular time intervals over 10 min, the membranes were rinsed with 2 ml of the respective wash solution, used for the preparation of the cell suspensions, and processed as outlined in Section 4.2.3. [^{14}C]sucrose uptake was expressed as μmol per mg of protein.

4.2.8. Protein assay

Protein concentrations were determined by the method of Lowry *et al.*, (1951). To a sample of 100 μl an equal volume of 1 M NaOH and 1 ml of an aqueous solution containing final concentrations of 2% (w/v) Na_2CO_3, 0.01% (w/v) $CuSO_4$ and 0.02% (w/v) sodium citrate was added. The mixture was

vortexed and kept at room temperature for 10 min before adding 100 µl of Folin & Ciocalteu's phenol reagent (diluted two fold in distilled water, BDH Chemicals Ltd., Poole, UK). The mixture was vortexed thoroughly and kept at room temperature for 60 min before reading the optical density at 600 nm. Standard curves ranging from 0 to 1 mg protein/ml were prepared using bovine serum albumin.

4.3. RESULTS AND DISCUSSION

4.3.1. Sugar competition studies on the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

E. coli JA221 did not take up sucrose (Fig. 4.1). [^{14}C]sucrose experiments with *E. coli* JA221 (pVS100) were complicated, since even well-washed mid-exponential cell samples contained extracellular sucrase (data not shown). This extracellular sucrase immediately degraded sucrose to glucose and fructose, which could be taken up by the cell's glucose and fructose transport systems. Washed *V. alginolyticus* cell samples did not contain extracellular sucrase. The addition of nonradioactive sucrose or sucrose plus glucose and fructose to *V. alginolyticus* and *E. coli* JA221 (pVS100) reduced the uptake of labelled sucrose by more than 90% (Fig. 4.1). The addition of nonradioactive glucose, fructose, or both reduced the uptake of [^{14}C]sucrose more in *E. coli* JA221 (pVS100) than in *V. alginolyticus* (compare Fig. 4.1 A and B), probably because the extracellular sucrase present in the resuspended

E. coli cells had hydrolyzed some of the [^{14}C]sucrose to glucose and fructose. It was concluded that sucrose was specifically taken up by *E. coli* JA221 (pVS100) and that the label did not enter primarily via the glucose and fructose uptake systems.

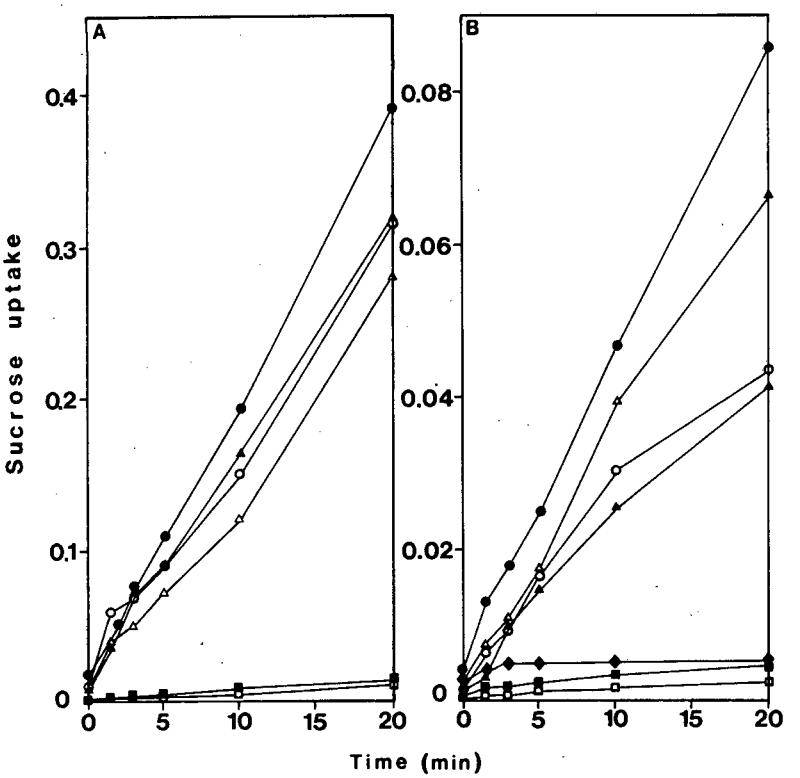

FIG. 4.1. [^{14}C]sucrose uptake (μmoles per mg of protein) by *V. alginolyticus* (A) and *E. coli* JA221 (pVS100) (B). Sucrose-induced washed cells were added to 0.5 mM [^{14}C]sucrose at time zero. Nonradioactive 15 mM sucrose (■), 28 mM fructose (△), 28 mM glucose (○), 28 mM glucose-28 mM fructose (▲), or 28 mM glucose-28 mM fructose-15 mM sucrose (□) was added at time zero. [^{14}C]sucrose uptake was determined after various time intervals. Other symbols: ●, no additions; ◆, [^{14}C]sucrose uptake by *E. coli* JA221.

4.3.2. Studies on the salt dependence of [^{14}C]sucrose uptake by *V. alginolyticus* and *E. coli* JA221 (pVS100)

Kakinuma and Unemoto (1985) reported that the sucrose uptake system in a marine *V. alginolyticus* strain required Na^+. The specific requirement for Na^+ could not be met by other cations (K^+, Li^+). The effect of Na^+, Li^+ and K^+ on sucrose uptake by the collagenolytic *V. alginolyticus* strain and *E. coli* JA221 (pVS100) was determined (Fig. 4.2).

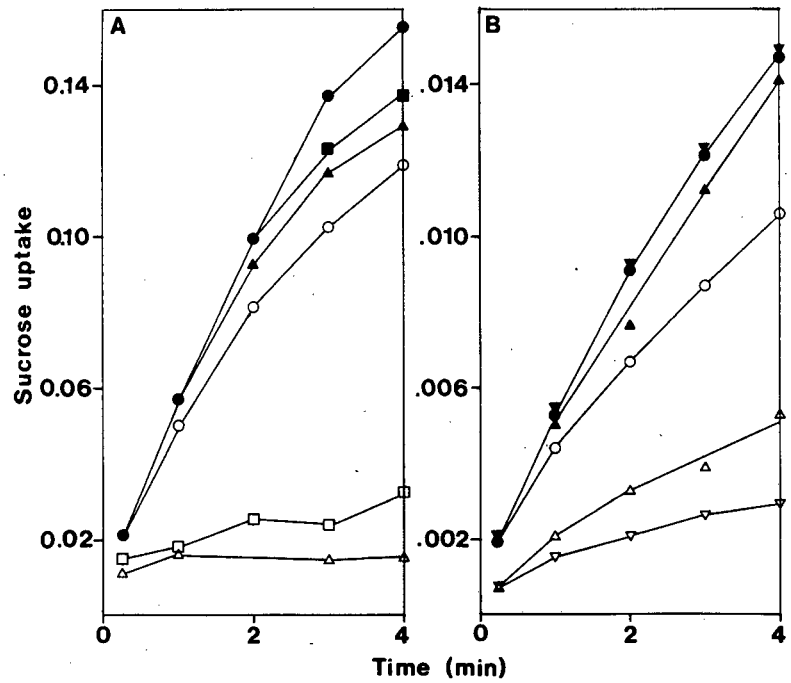

FIG. 4.2. Salt dependence of [^{14}C]sucrose uptake by *V. alginolyticus* (A) and *E. coli* JA221 (pVS100) (B). *V. alginolyticus* and *E. coli* cells were added to the incubation buffer containing 0.3 M or 0.1 M salts, respectively. CCCP (10 μM) was added 7 min prior to the addition of [^{14}C]sucrose. [^{14}C]sucrose uptake in *E. coli* was determined in the presence of 12.5 mM glucose and 12.5 mM fructose. [^{14}C]sucrose uptake is expressed in μmoles per mg of protein. Symbols: ● ,NaCl, KCl; ▲ , LiCl; ■ , choline chloride; ▼ , no salts; ○ ,methanol; △ , NaCl plus CCCP; □ , choline chloride plus CCCP; ▽ , no salts plus CCCP.

Sucrose uptake occurred in both *V. alginolyticus* and *E. coli* JA221 (pVS100) in both the presence or absence of Na^+. These strains also transported sucrose in the presence of K^+ and Li^+. The uptake of sucrose by *V. alginolyticus* and *E. coli* JA221 (pVS100) was markedly inhibited by the uncoupler CCCP (Fig. 4.2). Because CCCP was dissolved in methanol before being added to the cell sample, it could be argued that the methanol affected uptake, however, when methanol alone was added, sucrose uptake was only slightly inhibited.

4.3.3. Kinetic studies on the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

The rates of sucrose uptake were linear over 20 s, and the maximum amount of [^{14}C]sucrose taken up was less than 10% of the amount of [^{14}C]sucrose added. The maximum velocity (V_{max}) and apparent K_m value of sucrose uptake for the *V. alginolyticus* strain and *E. coli* JA221 (pVS100) were 130 nmol/mg of protein per min and 50 μM and 6 nmol/mg of protein per min and 275 μM, respectively (Fig. 4.3). The V_{max} for sucrose transport in *V. alginolyticus* is appreciably higher than in the transformed *E. coli* strain, and the K_m is lower.

4.3.4. Studies on the induction of the sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100)

The sucrose uptake system in *V. alginolyticus* and *E. coli* JA221 (pVS100) was induced by sucrose (Fig. 4.4). In induced *V. alginolyticus* cultures (propagated in sucrose

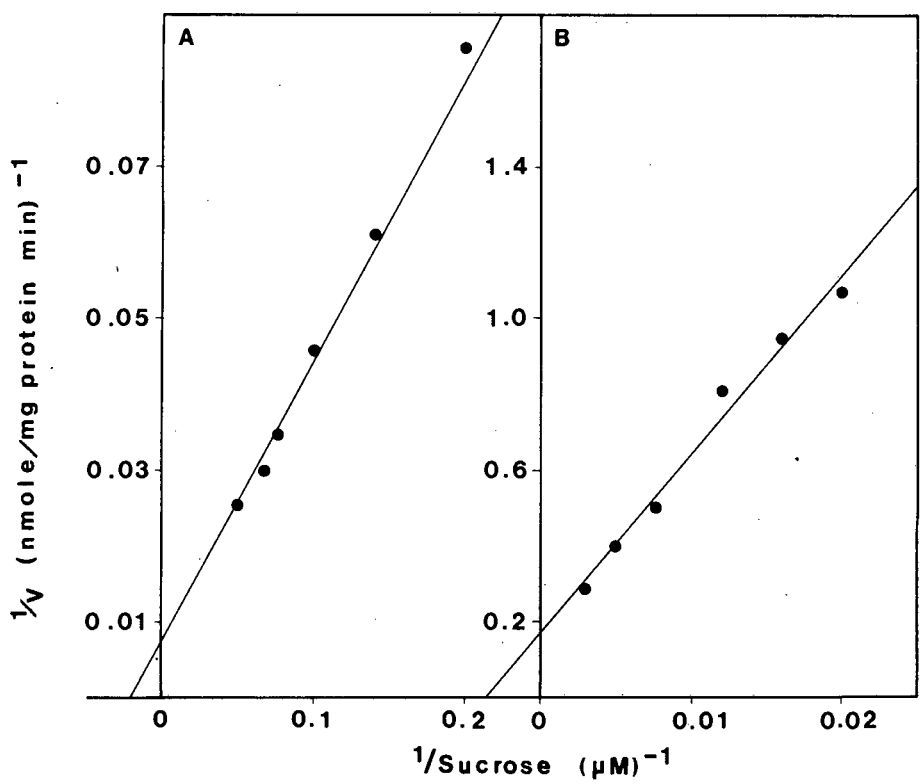

FIG. 4.3. Kinetic analysis of sucrose uptake. Lineweaver-Burk plots of [^{14}C]sucrose uptake in *V. alginolyticus* (A) and *E. coli* JA221 (pVS100) (B).

MM) [^{14}C]sucrose uptake was increased by a factor of 26 when compared to uninduced cultures grown in glucose MM. Under similar conditions, sucrose uptake in induced *E. coli* JA221 (pVS100) cultures increased by a factor of 8.

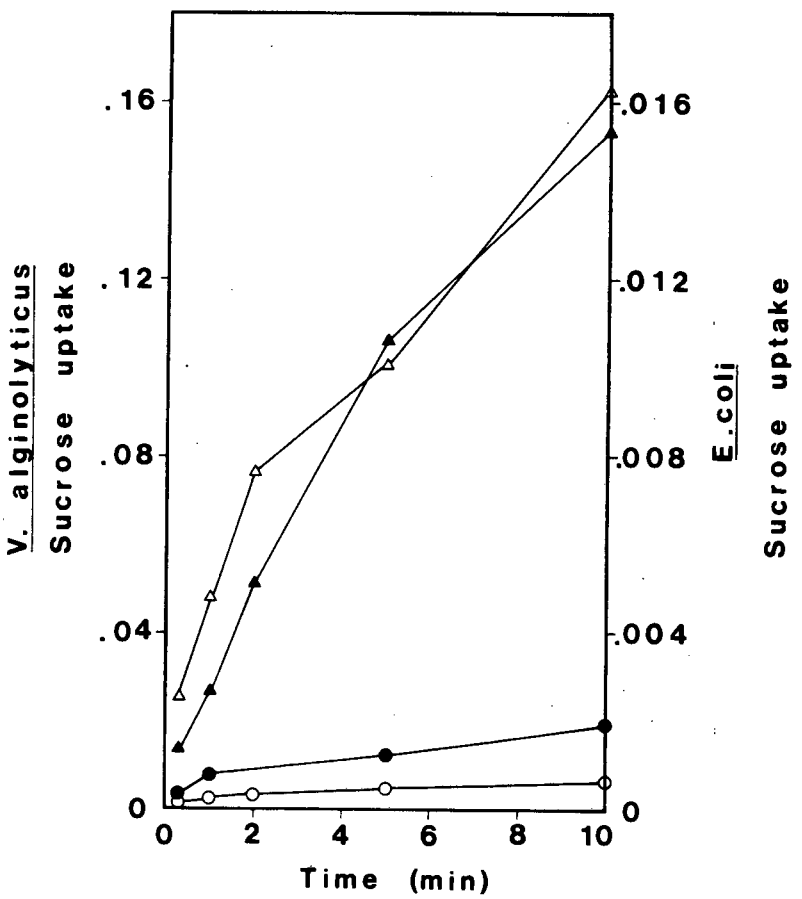

FIG. 4.4. Induction of [^{14}C]sucrose uptake in *V. alginolyticus* and *E. coli* JA221 (pVS100). *V. alginolyticus* cells grown in glucose (○) and sucrose (△) MM and *E. coli* JA221 (pVS100) cells grown in glucose (●) and sucrose (▲) MM were used. [^{14}C]sucrose uptake in *E. coli* was determined in the presence of 12.5 mM glucose-12.5 mM fructose. [^{14}C]sucrose uptake is expressed in μmoles per mg of protein.

4.4. CONCLUSION

The uptake studies indicated that the DNA fragment from *V. alginolyticus* which was cloned into *E. coli* JA221 contained the gene(s) required for the transport of sucrose. Both *V. alginolyticus* and *E. coli* JA221 (pVS100) actively transported sucrose by an inducible, Na^+-independent sucrose transport system. *E. coli* JA221 was not able to transport sucrose without the cloned DNA. It was concluded that in *E. coli* JA221 (pVS100) cells, sucrose was actively transported, following which it would be hydrolyzed to glucose and fructose by the intracellular sucrase. The extracellular sucrase also degraded the sucrose to glucose and fructose, which were taken up by the glucose and fructose transport systems. This dual system suggested that *E. coli* strains carrying pVS100 may be adept at scavenging and utilizing sucrose in low-grade industrial molasses media.

In view of the contradictory results obtained with respect to the salt requirements of the sucrose uptake system, it should be emphasized that the collagenolytic bacterium isolated from hides by Welton and Woods (1973; 1975) is without doubt a *V. alginolyticus* strain. Its initial classification as an *Achromobacter iophagus* strain was confirmed by the National Collection of Industrial Bacteria, Aberdeen, Scotland. Since then, the classification has been reinvestigated by M. Hendrie, National Collection of Industrial Bacteria, and the strain has been reclassified as *V. alginolyticus*. Recently, the 5S

rRNA sequence of the collagenolytic *V. alginolyticus* strain was shown to be similar to that of other marine *V. alginolyticus* strains (M. MacDonell, personal communication).

CHAPTER FIVE

MOLECULAR ANALYSIS OF THE *V. ALGINOLYTICUS SCRA* GENE

CHAPTER FIVE

MOLECULAR ANALYSIS OF THE *V. ALGINOLYTICUS SCRA* GENE

Summary: The nucleotide sequence of a 2.119 kb DNA fragment containing the *V. alginolyticus* sucrase gene (*scrA*) was determined. The complete amino acid sequence (484 residues) of the sucrase was deduced and significant homology was detected between the sucrase enzymes from *V. alginolyticus* and *B. subtilis*. The *V. alginolyticus* sucrase did not contain a classical N-terminal signal sequence. The codon usage in *scrA* of *V. alginolyticus* was very similar to other *Vibrio* genes and to *E. coli* derived genes. The *V. alginolyticus scrA* gene on the 2.119 kb DNA fragment was expressed from a putative secondary constitutive promoter in *E. coli*.

5.1. INTRODUCTION

It was briefly mentioned in Section 1.1 of the General Introduction, that *V. alginolyticus*, a gram-negative bacterium, resembles gram-positive *Bacillus* strains in a number of respects. This conclusion was based primarily on physiological studies, whereas no comparisons on a molecular level which support this statement have been reported to date.

Since the nucleotide sequence of the *B. subtilis* sucrase enzyme had been determined by Fouet *et al.*, (1986),

the generation of sequence data from the *V. alginolyticus* sucrase enzyme would allow such a comparison at a molecular level.

Moreover, homology studies on the deduced amino acid sequences from several sucrose hydrolyzing enzymes (*B. subtilis* levansucrase, levanase and sucrase and yeast invertase) (Steinmetz *et al.*, 1985; Martin *et al.*, 1987 b, Fouet *et al.*, 1986; Taussig and Carlson, 1983) might allow the identification of conserved motifs, possibly parts of functional domains.

The DNA sequence determination and molecular analysis of the *V. alginolyticus* sucrase gene and homology studies with other sucrose hydrolyzing enzymes will be discussed in this chapter.

5.2. MATERIALS AND METHODS

5.2.1. Bacterial strains, plasmids and phages

E. coli strains JA221 and LK111 and plasmid pVS100 have been described in Section 2.2.1. *E. coli* JM105, Δ*(lacproAB)*, thi, *strA*, *endA*, *sbcB*15, *hsdR*4, F'*traD*36, *proAB*, *lacI*^{q}ZΔM15 (Yanisch-Perron *et al.*, 1985) was used as the host strain for the generation of pVS107 single-stranded DNA. This strain was maintained on glucose MM (Appendix A.2) to ensure selection for the episome. The M13-derived Bluescript phagemid SK (Stratagene, San Diego) was used for the preparation of DNA sequencing templates. Single stranded DNA required for transcript mapping was obtained from the construct pVS107 prepared in the phage vector M13mp18 (Messing, 1983). Plasmids pVS104, pVS110, pVS111, pVS112, pVS113 and pVS107 were constructed in this study.

5.2.2. Standard methods, buffers and media

Appendix A lists standard techniques, buffers and media.

5.2.3. Subcloning of the sucrose utilization system

The 6.5 kb *Pst*I-*Hin*dIII fragment of pVS100 was gel purified and subcloned into *Pst*I-*Hin*dIII endonuclease digested Bluescript phagemid SK (Appendix A.1). The resulting construct was 9.4 kb in size and was designated pVS104. Deletions of the 2.7 kb *Hpa*I-*Hin*dIII and the 3.7 kb *Cla*I-*Cla*I fragments from pVS104 resulted in the generation of plasmids pVS110 and pVS111, respectively. In all

subcloning experiments, the DNA used was purified by isopycnic CsCl-EtdBr density gradient ultracentrifugation (Appendix A.1). Restriction endonuclease digestions were carried out as described in Appendix A.1. Apart from the gel purified DNA fragments, the endonuclease digestion products were purified by two phenol and two ether extractions followed by an ethanol precipitation in the presence of 0.3 M sodium acetate before being ligated and transformed into *E. coli* JA221 (Appendix A.1). Prior to ligating the *Hpa*I-*Hin*dIII endonuclease digested pVS104, the sticky ends generated by the restriction enzymes were filled in to allow blunt ended recircularization (Section 3.2.7). All transformants were selected on MacConkey agar containing Ap (100 μg/ml) (Appendix A.2).

5.2.4. Exonuclease III digestion

Progressive deletions from both the 5′ and 3′ ends of the insert were generated by unidirectionally digesting *Apa*I-*Sal*I and *Sac*I-*Bam*HI endonuclease digested pVS110, respectively, with exonuclease III (Henikoff, 1984) (Appendix A.1). DNA (5.5 μg), purified by isopycnic CsCl-EtdBr density gradient ultracentrifugation, was double digested with 10 units of each of the respective endonucleases (Appendix A.1). The digestion products were purified by two phenol and two ether extractions and were ethanol precipitated in the presence of 0.3 M sodium acetate. The precipitate was concentrated by centrifugation in an Eppendorf microfuge and stored dry.

For the exonuclease III digestion, the pellet was resuspended in 100 μl 1 x exobuffer (Appendix A.2) and

incubated at 37°C for 5 min before removing a time zero sample of 9 μl. 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 of 9 μl were removed at regular time intervals. All samples, including the time zero sample, were transferred immediately into 27 μl of SI mix containing 0.2 units/μl of SI nuclease (Boehringer Mannheim) in SI buffer (Appendix A.2) and were incubated for 30 min at room temperature. The SI nuclease digestion was terminated by the addition of 3.6 μl of SI stop buffer (Appendix A.2) followed by an incubation at 70°C for 10 min. Single stranded ends, generated by non specific SI nuclease digestion, were filled in by adding to each sample tube 3.6 μl of Klenow mix containing approximately 0.15 units/μl Klenow enzyme (Boehringer Mannheim) diluted in Klenow buffer (Appendix A.2). The mixtures were incubated at room temperature for 3 min before the addition of 75 μM of each dNTP. The filling-in reactions were allowed to proceed for 5 min at room temperature after which the DNA fragments were purified by two phenol and two ether extractions and ethanol precipitated in the presence of 0.3 M sodium acetate. The pellet was resuspended in TE buffer (Appendix A.2) and the shortened DNA fragments were blunt-end ligated as described in Appendix A.1. The deletions were transformed into *E. coli* LK111 (Appendix A.1) and transformants were selected on LB agar containing Ap (100 μg/ml). Useful DNA sequencing templates were selected after sizing shortened pVS110 plasmids isolated by the small-scale procedure (Appendix A.1).

5.2.5. Nucleotide sequencing

The nt sequence of both strands of the sucrase gene was determined using overlapping DNA fragments generated by exonuclease III digestion of pVS110. DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method described in Section A.1.12 using the M13 universal primer included in the Sequenase DNA Sequencing kit or the M13 reverse primer (Code N 4512) supplied by Amersham.

The DNA and deduced aa sequences were analysed on an IBM XT computer using the Genepro programme version 4.1 (Riverside Scientific). The databases GenBank, European Molecular Biology Laboratory (EMBL) and Protein Identification Resource (PIR) of June 1988 were screened for related DNA and protein sequences. Sequence alignments and secondary structure predictions were performed using Genepro and Microgenie (September 1986, Beckman Instruments), respectively.

5.2.6. Transcript mapping

5.2.6.1. Isolation of RNA

Total cellular RNA was extracted by a modification of the method described by Aiba *et al.*, (1981). Gloves were worn at all times during the RNA extraction procedure and disposable equipment was used whenever possible. All equipment was soaked for 14 h at 37^{o}C in DEPC water (0.1% (v/v) diethylpyrocarbonate) before being autoclaved. All solutions, except for those containing Tris, were prepared

in DEPC water and sterilized by autoclaving. Tris-containing solutions were prepared in distilled water.

Early-exponential-phase cultures of *E. coli* JA221 (SK) and JA221 (pVS110) were harvested by centrifugation at 2000 x g for 10 min at room temperature and were resuspended in 10 ml dissociation buffer (0.1 M Tris-HCl pH 8.0, 1% (w/v) SDS and 1 mM EDTA). Phenol (1.5 ml), preheated to 60°C and an equal volume of chloroform:isoamylalcohol (24:1 v/v) were added to the lysed cells and kept for 5 min at 60°C with gentle agitation. The phases were separated by centrifugation at 2000 x g for 5 min at room temperature, the upper phase was removed and was phenol extracted as described above until no more protein was visible at the interface. The aqueous phase obtained after the last in the series of phenol extractions was subsequently twice extracted with an equal volume of ice cold water saturated ether. Any remaining traces of ether were evaporated off at 60°C before the nucleic acids were precipitated in 2.5 x volume absolute ethanol containing 0.12 M sodium acetate. The mixture was kept at -20°C for 14 h before the precipitate was pelleted at 2000 x g for 30 min at 4°C and washed with 0.5 ml of 70% ethanol. The pellet was dried and resuspended in 1 ml of 100 mM sodium acetate, 5 mM $MgSO_4$ containing 70 units RNase free DNaseI (Boehringer Mannheim). This mixture was kept at room temperature for 45 min after which it was reprecipitated with ethanol as described above and resuspended in 0.5 ml sterile distilled DEPC water. The quality of the RNA preparation was assessed by resolving a volume of 10 μl on a Tris-acetate-buffered 1% agarose gel.

The concentration of RNA was determined spectrophotometrically. One absorbance unit (260 nm) was considered equivalent to 36 μg RNA/ml.

5.2.6.2. Construction of pVS107 and preparation of single-stranded DNA

Plasmid pVS112, representing the shortest sucrase-positive exonuclease III shortened product of pVS110, was double digested with the restriction endonucleases *Kpn*I and *Xba*I (Appendix A.1). The resulting 2.6 kb fragment containing the *scrA* gene was ligated to *Kpn*I-*Xba*I endonuclease digested M13mp18 phage vector (Messing, 1983) (Appendix A.1) after both digestion products were twice phenol and ether extracted and ethanol precipitated in the presence of 0.3 M sodium acetate. The ligation products were transformed into competent *E. coli* JM105 (Appendix A.1). Instead of allowing the expression of the transferred DNA by incubating the transformation mixture in 400 μl of LB broth at 37°C for 60 min, the entire transformation mix was added to 3 ml H top agar (Appendix A.2) maintained at 42°C. The H top agar contained 15 μl of 100 mM IPTG, 40 μl of X-gal (2% in dimethylformamide) and 200 μl of a fresh, early-exponential-phase culture of *E. coli* JM105. The H top agar was well mixed before being poured evenly over a prewarmed (37°C) H agar plate (Appendix A.2). After incubating the plates for 14 h at 37°C several randomly chosen white plaques were removed and inoculated into 1.5 ml of a 100-fold diluted *E. coli* JM105 late stationary culture. The cultures were incubated for 6 h at 38°C with vigorous shaking before being harvested by centrifugation for 5 min

at room temperature in an Eppendorf microfuge. The supernatant was transferred into a fresh tube, taking extreme care not to transfer any cells, and stored at 4°C before being processed further. The replicative form of the recombinant phagemid was isolated from the cell pellet by the small-scale plasmid isolation procedure described in Section A.1.2. Diagnostic endonuclease digestions were performed on the plasmid preparation (Appendix A.1) so as to identify the desired recombinant phagemid pVS107.

The supernatants containing the single-stranded form of pVS107 which were stored at 4°C were recentrifuged and transferred into a fresh tube to ensure the removal of all cells. The supernatant was subsequently added to 200 µl PEG/NaCl (20% (w/v) polyethylene glycol 6000, 2.5 M NaCl), and the mixture was shaken and left to stand at room temperature for 15 min. The viral pellet was recovered by repeated centrifugation in an Eppendorf microfuge, removing the supernatant and any traces of PEG after each centrifugation step. After resuspending the pellet in 100 µl of TE buffer (Appendix A.2), 50 µl of phenol was added to the suspension upon which it was vortexed for 20 sec and held at room temperature for 15 min. The phases were separated by centrifugation for 3 min in an Eppendorf microfuge and the upper aqueous phase was transferred into a fresh microcentrifuge tube. The nucleic acids suspended in the aqueous phase were ethanol precipitated in the presence of 0.3 M sodium acetate for 5 min at -70°C. The single-stranded DNA was recovered by centrifugation for 10 min in an Eppendorf microfuge and the pellet was washed with 1 ml

cold (-20°C) absolute ethanol before being dried. The dry pellet was resuspended in 15 to 50 µl of TE buffer (Appendix A.2) and stored at -20°C.

5.2.6.3. Preparation of the strand-specific probe

A strand-specific probe for SI nuclease mapping was prepared from the single-stranded form of pVS107 which corresponded to the non-coding strand of the *scrA* gene. A 15-mer synthetic primer (Vasucl) was synthesized by D. Botes (Department of Biochemistry, University of Cape Town, SA) in a Beckman System 1 DNA Synthesizer (version 2.1). Primer Vasucl had the sequence 5' ATATCTTTGCTCTAC 3' which corresponded to positions +25 to +39 of the sucrase structural gene (Fig. 5.3).

Primer Vasucl (9 ng) was added to 5 ng of single-stranded pVS107 and 3 µl of 5 x Sequenase buffer supplied in the Sequenase DNA Sequencing kit (Section A.1.12). The annealing reaction was carried out by heating the mixture for 2 min to 65°C and allowing it to cool down gradually to ambient temperature over 30 min. The strand specific probe was prepared in the presence of 20 µCi [α-^{32}P]dCTP (specific activity 3000 Ci/mmole) essentially as described for sequenase labelling reactions. The annealed primer was extended for 2 min at room temperature in the presence of 0.2 µM (final concentration) of a mixture containing all dNTP except for dCTP. After the addition of 1.5 µl of a 10 µM dNTP mixture (as above) the enzyme reaction was allowed to continue for a further 3 min before adding 1.5 µl of a 100 µM dNTP mixture (including dCTP). After 5 min the reaction was terminated by the addition of 1.5 µl of 0.5 M

EDTA (pH 8.0) and the reaction products were stored at -20°C. The size of the probe was estimated from a 6% (w/v) denaturing polyacrylamide gel.

5.2.6.4. Transcript mapping by SI nuclease protection

The RNA preparations, which were resuspended in 0.5 ml of sterile, distilled DEPC water were reprecipitated in 2.5 x volume of absolute ethanol in the presence of 0.3 M sodium acetate (see Section 5.2.6.1) and resuspended in hybridization buffer (20 mM HEPES pH 6.5, 0.4 M NaCl, 80% formamide; Dixon, 1984) so as to obtain a final concentration of 6.7 μg RNA/μl. A volume of 30 μl of this mixture was heated to 80°C for 10 min after which 2 μl of the probe was added. The hybridization reaction was allowed to proceed at 50°C and 40°C for 3 h at each temperature. The mixture was diluted 10-fold with buffer (30 mM sodium acetate pH 4.5, 3 mM zinc chloride, 300 mM NaCl; Dixon, 1984) and nucleic acids were digested with 1 unit/μl of SI nuclease (Boehringer Mannheim) at 37°C for 30 min in the presence of 6 ng/μl of viral RNA. The digestion products were phenol and ether extracted, ethanol precipitated in the presence of 0.3 M sodium acetate, dried and resuspended in 4 μl of sterile, distilled DEPC water and an equal volume of tracking dye (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol). A sample of 1.2 μl was analysed on a 6% (w/v) polyacrylamide gel using [α-^{32}P]dCTP labelled dideoxy sequencing products of pVS110 DNA primed with the Vasuc1 primer as size markers.

5.2.7. Sucrase assays and uptake of [^{14}C]sucrose

Competent *E. coli* JA221 cells were transformed with plasmid preparations of all the exonuclease III digestion products of pVS110 as well as plasmids pVS104, pVS110 and pVS111 (Appendix A.1). Cultures (10 ml) of all *E. coli* JA221 transformants were grown overnight in sucrose MM at 37^{o}C. Sucrase assays were carried out on sonicated cell extracts prepared from all cultures as described in Section 2.2.8.

[^{14}C]sucrose uptake of *E. coli* JA221 cells containing plasmids pVS104, pVS110, pVS111, pVS112 or pVS113 was determined as outlined in Section 4.2.4. The uptake reactions were started by the addition of 250 μM [^{14}C]sucrose (final concentration, specific activity 10 Ci/mol, Amersham) to 400 μl of cells and samples were withdrawn at regular time intervals over 21 min.

5.3. RESULTS AND DISCUSSION

5.3.1. Subcloning of the sucrose utilization system

Plasmid pVS100 contained the genes from *V. alginolyticus* coding for both sucrase and sucrose uptake activity in transformed *E. coli* JA221 as described in Chapters 3 and 4. Progressively larger deletions from the 5′ end of the insert in pVS100 resulted in the loss of sucrose uptake activity whereas the sucrase activity was retained (Fig. 5.1). Further deletions generated by exonuclease III digestion of pVS110 finally resulted in the

loss of both activities. Two exonuclease III shortened products of pVS110, namely pVS112 and pVS113, represented the shortest and longest sucrase-positive and sucrase-negative fragment, respectively.

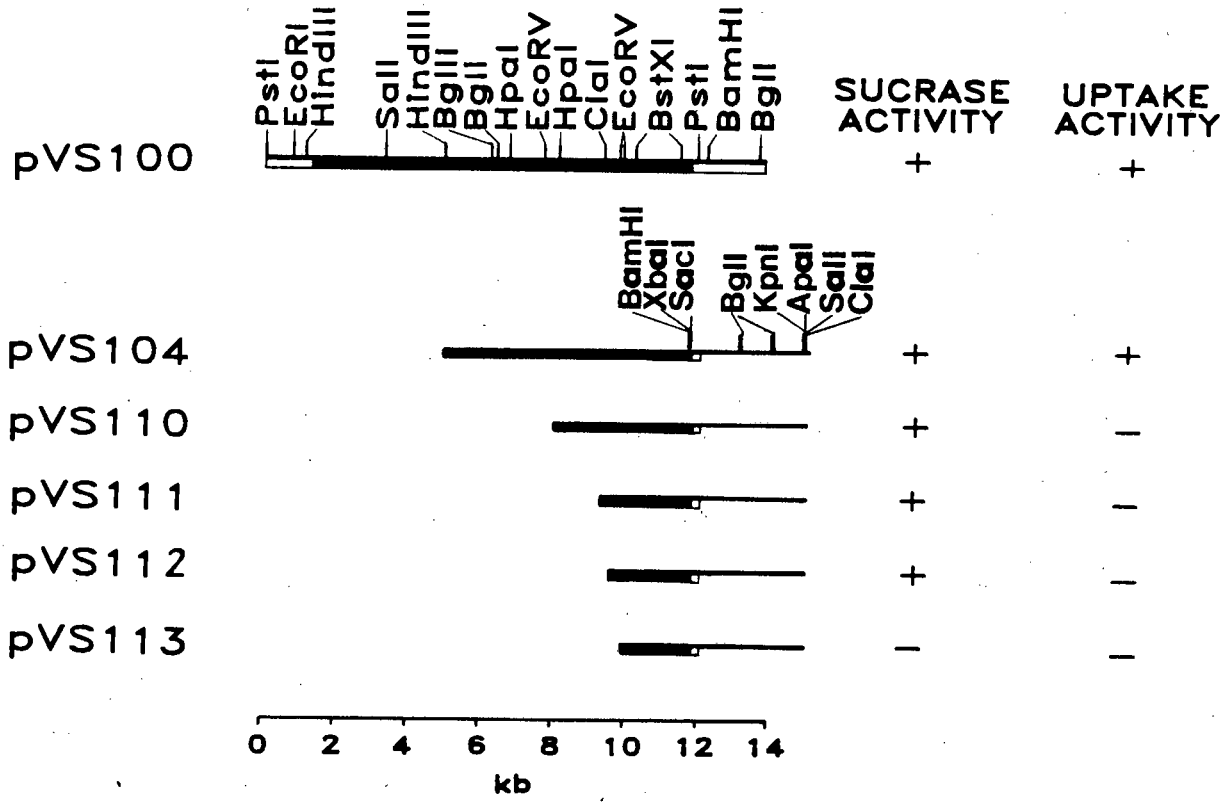

FIG. 5.1. Restriction endonuclease map, subclones and deletion derivatives of pVS100. The thin lines and the open and closed boxes represent Bluescript vector DNA, pEcoR251 vector DNA and *V. alginolyticus* DNA, respectively. Sucrase and sucrose uptake activities are indicated for each construct.

5.3.2. Nucleotide sequence of the coding region of the *V. alginolyticus* sucrase gene

DNA sequencing templates generated by exonuclease III digestion from the 5′ and 3′ ends of the insert in plasmid pVS110 were constructed. Overlapping sequence data were obtained by the chain termination method of Sanger *et al.*, (1977) of both DNA strands encompassing the region of the *scrA* gene (Fig. 5.2).

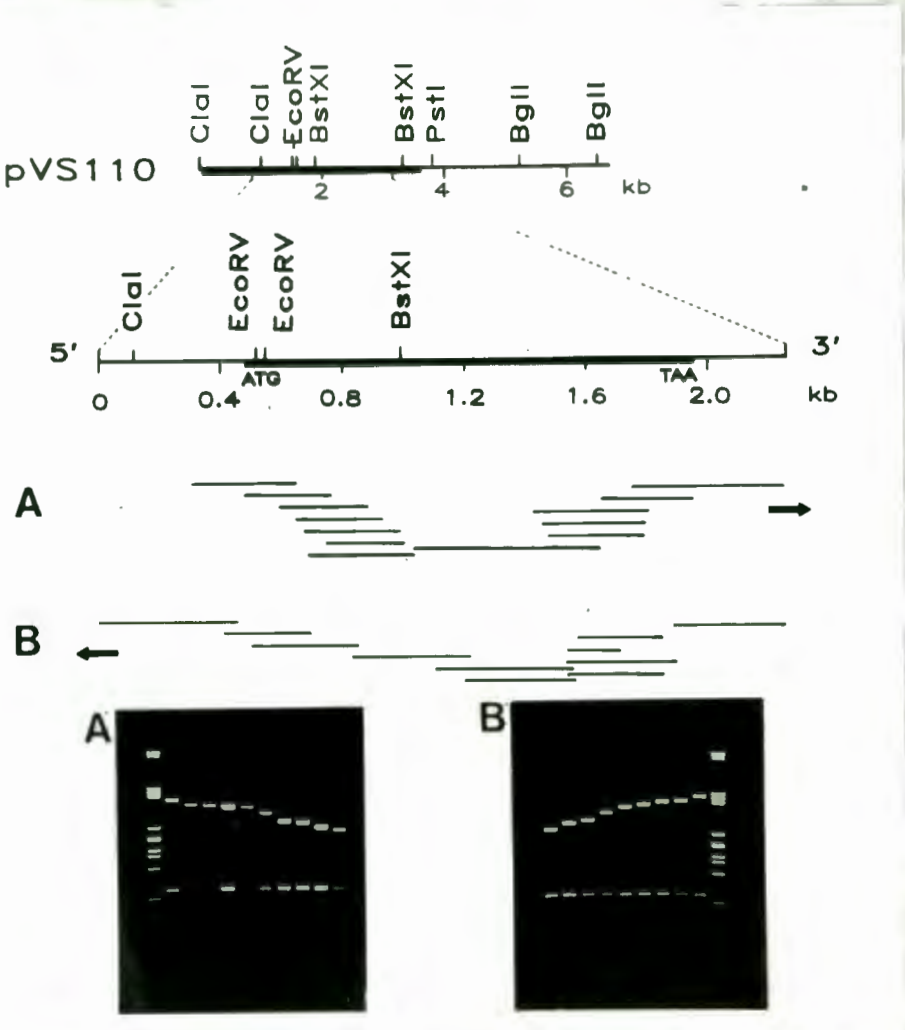

FIG. 5.2. Nucleotide sequencing strategy for the *V. alginolyticus scrA* gene. For plasmid pVS110, the bold and thin lines denote *V. alginolyticus* DNA and vector DNA, respectively. In the enlarged section of pVS110, the ORF of the *scrA* gene is indicated by a thick line, beginning and ending with an ATG and TAA codon, respectively. The ethidium bromide stained agarose gels, A′ and B′, contain *Bgl*I endonuclease digested DNA sequencing templates generated by exonuclease III digestion of plasmid pVS110, with *Pst*I endonuclease digested λDNA size markers at the extreme ends. The thin lines (A and B) represent the extent and direction of sequence information obtained from each corresponding template.

The nt sequence of the 2.119 kb DNA fragment coding for the *scrA* gene contained an ORF which from the presumptive start codon (ATG) to the stop codon (TAA) contained 1452 nt encoding 484 aa residues (Fig. 5.3). The M_r of the predicted polypeptide encoded by this ORF was 55 657. Cell-free coupled transcription and translation studies described in Chapter 2 (Fig. 2.4) indicated that pVS100, containing the entire *V. alginolyticus* sucrose utilization system, produced three polypeptides. It was considered to be likely that the polypeptide with an apparent M_r of approximately 58 000 was the sucrase enzyme. Since pVS100 produced three polypeptides, the two polypeptides with apparent M_r of 50 000 and 32 000 might be components of the sucrose uptake system.

Fig. 5.4 depicts the hydropathicity profile of the *V. alginolyticus* sucrase enzyme (Kyte and Doolittle, 1982). The results obtained from λ:Tn*phoA* mutagenesis, which were described in Chapter 3, led to the conclusion that the *V. alginolyticus* sucrase did not contain a N-terminal signal sequence. This suggestion was supported by the absence of a typical signal sequence consisting of a short stretch of positively charged aa followed by a core of hydrophobic aa at the N-terminal region of the *V. alginolyticus* sucrase (Von Heijne, 1983). Instead, the first 37 aa residues of the N-terminus of the sucrase were hydrophilic (Fig. 5.4).

V. alginolyticus DNA has a G+C content of 45-47% (Krieg and Holt, 1984) and the G+C content of 43.9% of the *scrA* structural gene reflected the G+C content of the bacterial DNA. The percentage synonymic codon usage of the *scrA* gene

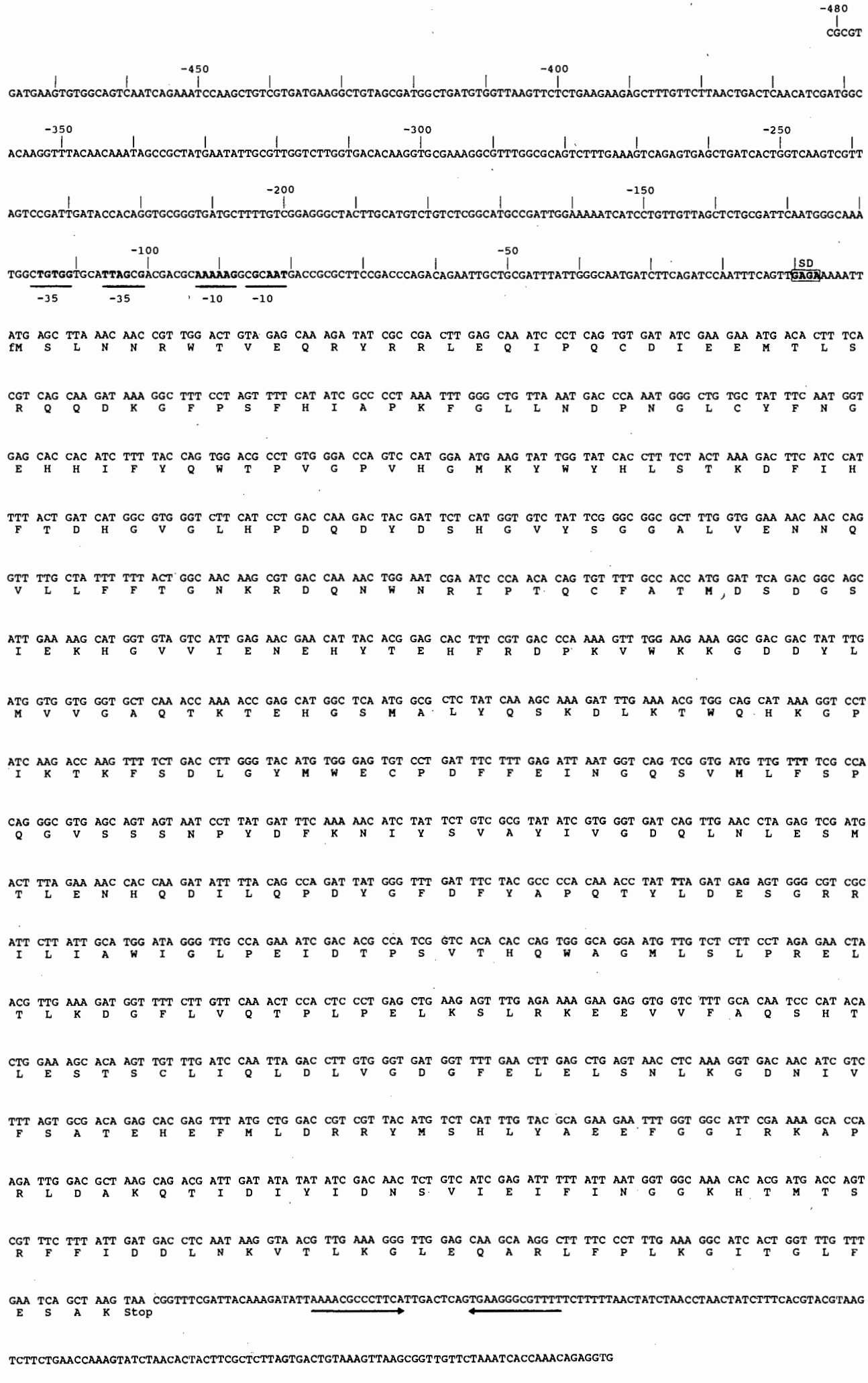

FIG. 5.3. **Legend: see next page**

FIG. 5.3. Complete nucleotide sequence of the *scrA* structural gene and flanking regions from *V. alginolyticus*. The deduced amino acid sequence is given in single-letter code from position 1 to 1452 (484 residues). The -10 and -35 regions of the putative promoters are in boldface type and underlined. The putative ribosome binding site is boxed. The complementary invert repeats starting at position 1478, representing the transcriptional terminator, are indicated by arrows.

was compared to that of the following genes: *V. alginolyticus* exoprotease A (S. Deane, personal communication), *V. alginolyticus glnA* (R. Maharaj, personal communication), *Vibrio cholerae ctx* (Mekalanos *et al.*, 1983), *Vibrio harveyi luxA* and *luxB* (Cohn *et al.*, 1985; Johnston *et al.*, 1986) and an average of 25 *E. coli* genes (Konigsberg and Godson, 1983) (Table 5.1). Apart from a few exceptions, the percentage synonymic use of each codon in the *scrA* gene reflected the bias of the other *Vibrio* genes as well as the bias observed for *E. coli* derived genes. This was expected considering the close relatedness and similarity in %G+C content between *Vibrio* strains and *E. coli* (Krieg and Holt, 1984). The preferred codon usages reported for *E. coli* for leucine (CTG), proline (CCG) and glutamine (CAG) were not observed for genes from *Vibrio*. Instead leucine was encoded at equal frequency by all six of the possible leucine codons, proline showed preference for CCT and CCA codons, and glutamine was preferentially encoded by CAA.

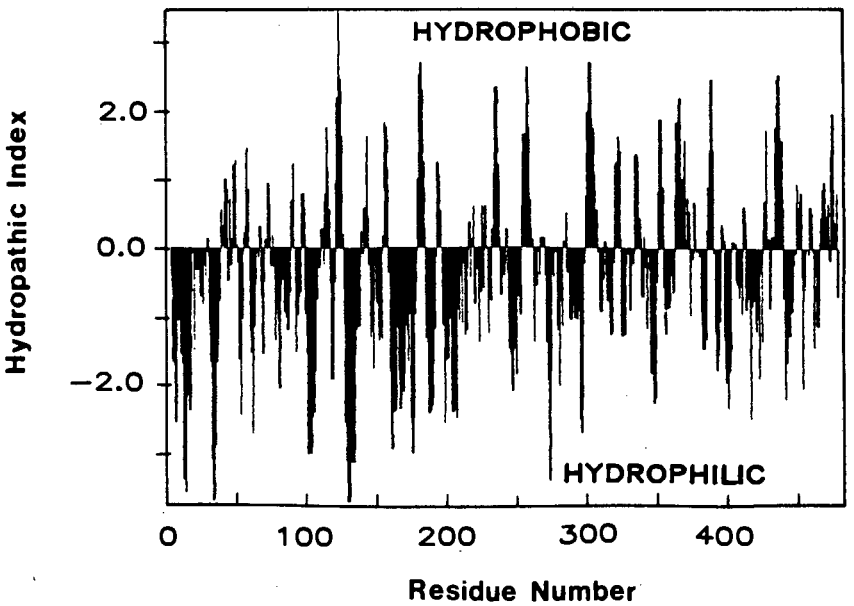

FIG. 5.4. Hydropathicity plot of the *V. alginolyticus* sucrase. The window size chosen was five amino acid residues.

TABLE 5.1. Codon usage in six *Vibrio* genes compared with that in 25 *E. coli* genes[a]

Residue and codon	*Vibrios*								*E.c*
	scrA	*glnA*	*proA*	*luxA*	*luxB*	*ctx*	Tc	T% su	T% su
Phe UUU	22(76)	7(27)	11(73)	7(78)	8(47)	9(75)	64	59	44
Phe UUC	7(24)	19(73)	4(27)	2(22)	9(53)	3(25)	44	41	56
Leu UUA	6(13)	1(3)	8(22)	5(18)	3(11)	8(50)	31	17	6
Leu UUG	17(36)	0(0)	8(22)	8(29)	9(31)	3(19)	45	24	8
Leu CUU	11(23)	10(35)	6(17)	4(14)	3(11)	4(25)	38	21	9
Leu CUC	4(9)	0(0)	5(14)	5(18)	3(11)	0(0)	17	9	7
Leu CUA	3(6)	16(55)	6(17)	2(7)	7(25)	1(6)	35	19	2
Leu CUG	6(13)	2(7)	3(8)	4(14)	3(11)	0(0)	18	10	69
Ile AUU	11(39)	3(12)	8(40)	4(33)	12(55)	7(41)	45	36	37
Ile AUC	15(54)	23(88)	9(45)	8(67)	10(45)	2(12)	67	54	62
Ile AUA	2(7)	0(0)	3(15)	0(0)	0(0)	8(47)	13	10	1
Met AUG	13 -	19 -	10 -	4 -	9 -	4 -	59	-	-
Val GUU	3(13)	16(47)	10(20)	6(35)	6(32)	7(54)	48	31	38
Val GUC	8(33)	0(0)	11(22)	5(29)	5(26)	0(0)	29	18	13
Val GUA	3(13)	13(38)	11(22)	1(7)	3(16)	3(23)	34	22	23
Val GUG	10(41)	5(15)	17(36)	5(29)	5(26)	3(23)	45	29	27

TABLE 5.1. (continued)

Ser UCU	7(23)	16(50)	10(15)	5(33)	7(41)	6(26)	51	27	27
Ser UCC	1(3)	0(0)	5(7)	2(13)	1(6)	2(9)	11	6	26
Ser UCA	4(13)	11(35)	7(10)	1(7)	5(29)	7(30)	35	19	8
Ser UCG	5(16)	3(9)	13(19)	1(7)	1(6)	1(4)	24	13	11
Ser AGU	9(29)	0(0)	16(24)	3(20)	0(0)	7(31)	35	19	6
Ser AGC	5(16)	2(6)	17(25)	3(20)	3(18)	0(0)	30	16	2
Pro CCU	11(50)	6(25)	5(23)	3(60)	4(33)	4(28)	33	33	9
Pro CCC	0(0)	0(0)	2(9)	0(0)	0(0)	1(7)	3	3	6
Pro CCA	11(50)	16(67)	10(45)	2(40)	5(42)	5(36)	49	50	20
Pro CCG	0(0)	2(8)	5(23)	0(0)	3(25)	4(29)	14	14	65
Thr ACU	7(26)	6(29)	5(18)	5(31)	7(33)	6(60)	36	29	24
Thr ACC	6(22)	1(4)	9(30)	6(37)	5(24)	2(20)	29	23	51
Thr ACA	6(22)	10(48)	8(26)	2(13)	5(24)	1(10)	32	26	6
Thr ACG	8(30)	4(19)	8(26)	3(19)	4(19)	1(10)	28	22	20
Ala GCU	4(25)	16(34)	10(19)	3(19)	5(19)	4(27)	42	24	28
Ala GCC	3(19)	0(0)	8(15)	5(31)	5(19)	2(13)	23	13	19
Ala GCA	6(37)	22(47)	17(33)	2(13)	7(27)	9(60)	63	37	23
Ala GCG	3(19)	9(19)	17(33)	6(37)	9(35)	0(0)	44	26	30
Tyr UAU	13(65)	0(0)	13(59)	3(50)	6(38)	12(67)	47	48	41
Tyr UAC	7(35)	16(100)	9(41)	3(50)	10(62)	6(33)	51	52	59
His CAU	12(60)	0(0)	3(43)	5(71)	5(45)	9(82)	34	49	39
His CAC	8(40)	13(100)	4(57)	2(29)	6(55)	2(18)	35	51	61

TABLE 5.1. (continued)

Gln	CAA	13(52)	7(78)	25(93)	4(80)	7(54)	9(75)	65	71	27
Gln	CAG	12(48)	2(22)	2(7)	1(20)	6(46)	3(25)	26	29	73
Asn	AAU	8(38)	0(0)	10(37)	4(40)	9(47)	7(64)	38	36	24
Asn	AAC	13(62)	17(100)	17(63)	6(60)	10(53)	4(36)	67	64	76
Lys	AAA	17(63)	18(72)	7(64)	5(56)	14(70)	4(50)	65	65	77
Lys	AAG	10(37)	7(28)	4(36)	4(44)	6(30)	4(50)	35	35	23
Asp	GAU	17(50)	10(26)	20(51)	11(61)	14(50)	17(89)	89	51	51
Asp	GAC	17(50)	28(74)	19(49)	7(39)	14(50)	2(11)	87	49	49
Glu	GAA	14(45)	27(79)	5(38)	13(76)	18(75)	8(73)	85	65	73
Glu	GAG	17(55)	7(21)	8(62)	4(24)	6(25)	3(27)	45	35	27
Cys	UGU	4(80)	2(66)	9(82)	0(0)	7(88)	1(50)	23	72	42
Cys	UGC	1(20)	1(34)	2(18)	3(100)	1(12)	1(50)	9	28	58
Trp	UGG	9 -	2 -	5 -	3 -	6 -	3 -	28	-	-
Arg	CGU	8(44)	13(68)	5(29)	5(63)	4(31)	1(6)	36	39	58
Arg	CGC	2(11)	6(32)	6(35)	3(37)	3(23)	0(0)	20	22	35
Arg	CGA	3(17)	0(0)	3(18)	0(0)	5(38)	2(12)	13	14	2
Arg	CGG	0(0)	0(0)	0(0)	0(0)	0(0)	1(5)	1	1	3
Arg	AGA	4(22)	0(0)	3(18)	0(0)	1(8)	10(59)	18	20	1
Arg	AGG	1(6)	0(0)	0(0)	0(0)	0(0)	3(18)	4	4	0

TABLE 5.1. (continued)

Gly GGU	15(41)	25(74)	25(47)	5(42)	12(46)	10(45)	92	50	48
Gly GGC	12(32)	9(26)	16(30)	5(42)	9(35)	2(10)	53	29	41
Gly GGA	3(8)	0(0)	7(13)	1(8)	2(8)	6(27)	19	10	5
Gly GGG	7(19)	0(0)	5(10)	1(8)	3(11)	4(18)	20	11	7

[a] The *Vibrio* derived genes listed in Table 5.1 are the *V. alginolyticus* sucrase (*scrA*), *V. alginolyticus* glutamine synthetase (*glnA*), *V. alginolyticus* exoprotease A (*proA*), *V. harveyi* luciferase α-subunit (*luxA*), *V. harveyi* luciferase β-subunit (*luxB*), and *V. cholerae* toxin (*ctx*). The codon usage for *E. coli* (*E.c*) was derived from a compilation of 25 sequenced, nonregulatory *E. coli* genes (Konigsberg and Godson, 1983). The numbers listed for each codon represent the total number of times this codon had been used in each repective protein with the corresponding percentage synonymic use given in brackets. The percentage synonymic use of each codon was obtained by dividing the number of times a codon was used in each gene by the number of times all of the codons, specifying the same amino acid, were used expressed as a percentage. Tc, total number of codons; T%su, total percentage synonymic use.

5.3.3. Analysis of the upstream and downstream regions of the *scrA* gene

Normally ORF's are preceded by a ribosome binding site (Watson *et al.*, 1987; Shine and Dalgarno, 1974) situated between 8 and 13 nt upstream from the translation initiation codon ATG. A conventional ribosome binding site was not present upstream of the start codon of the *scrA* gene. A GAGA nt sequence was situated between -7 and -10 nt from the *scrA* initiation codon (Fig. 5.3).

Analysis of the 481 nt upstream of the *scrA* gene did not show the presence of putative promoter sequences with strong homology to the -10 (TATAAT) and -35 (TTGACA) RNA polymerase binding consensus sequences (Rosenberg and Court,

1979; Hawley and McClure, 1983). However, SI nuclease mapping performed on total cellular RNA extracted from *E. coli* JA221 containing plasmid pVS110 indicated a transcriptional start site at nt -77 or nt -78 (Fig. 5.5). RNA extracted from control *E. coli* JA221 cells harbouring the Bluescript vector did not produce a hybridization band at the equivalent position (Fig. 5.5).

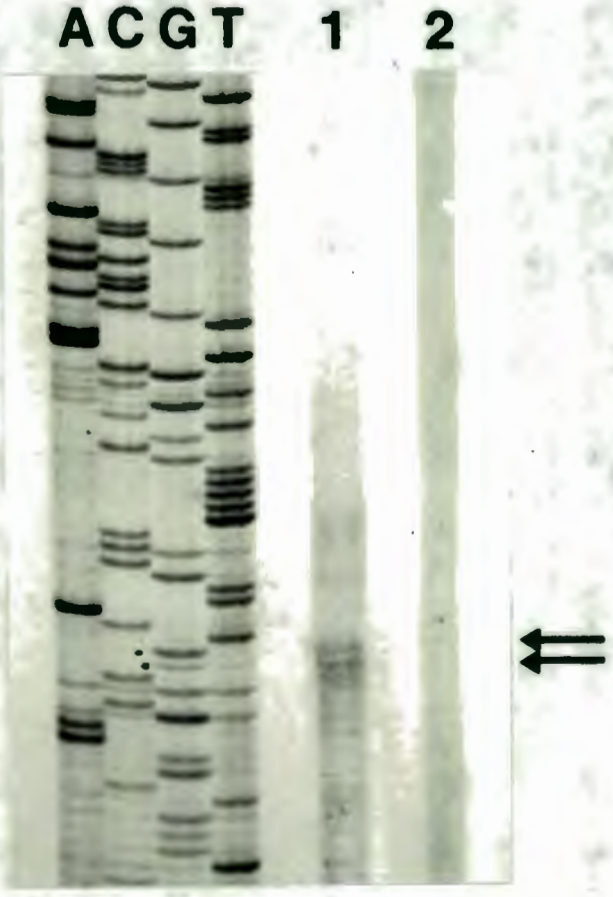

FIG. 5.5. Identification of the transcriptional start of the *scrA* gene from *V. alginolyticus*. Lanes on the autoradiogram labelled A, C, G and T represent the dideoxy sequencing reaction products of pVS110 DNA primed with the synthetic primer Vasuc1. Lanes 1 and 2 contain the protected RNA-DNA hybrid fragments (indicated by arrows) after SI nuclease digestion (1.0 unit/ μl) of the [α-^{32}P]dCTP-labelled probe hybridized to total cellular RNA extracted from *E. coli* JA221 containing plasmid pVS110 and the Bluescript vector SK, respectively.

Analysis of the nt sequence immediately adjacent to the transcriptional start site indicated that there were two possible putative promoter sequences with low homology to the conventional -10 and -35 consensus sequences. The first putative promoter sequence (-81 to -86 and -101 to -106) showed some homology to the -10 and -35 consensus regions but the spacing between the -10 and -35 regions was not optimal. The second putative promoter sequence (-88 to -93 and -111 to -116) was characterized by correct spacing but low -10 and -35 homology. Although SI nuclease mapping confirmed that the *scrA* gene of pVS110, which did not contain a functional sucrose uptake system (Fig. 5.1), was expressed from a promoter region within the cloned *V. alginolyticus* DNA, it was considered unlikely to be the promoter utilized by the entire *V. alginolyticus* sucrose uptake region. In Chapters 3 and 4 it was shown that sucrose uptake and sucrase activity were inducible in pVS100 and that sucrase activity was not detected in *E. coli* (pVS100) cultures grown in glucose MM. Sucrase production by pVS100 in *E. coli* was also subject to glucose repression which was relieved by cAMP. However, sucrase production by pVS110 in *E. coli* was constitutive and was detected in cultures grown in glucose MM (G. Blatch, personal communication). A CAP binding site (Ebright, 1982) was not identified in the 481 nt region upstream of the *scrA* gene.

Since the constitutive promoter adjacent to the *V. alginolyticus scrA* gene was not the inducible promoter utilized by the entire sucrose utilization region, it is possible that the sucrose uptake gene(s) and *scrA* gene are

located in an operon with a regulatory region upstream of the sucrose uptake gene(s).

A potential rho-independent transcriptional termination loop (Watson *et al.*, 1987) was situated 22 nt downstream from the TAA translational stop codon (Figs. 5.3 and 5.6). It was characterized by two 13 nt complementary invert repeats which, when transcribed, would have the potential to form a stem-loop structure with a ΔG=-23.2 kcal/mole (Salser, 1977). The invert repeats were directly followed by the nt sequence 5′ TCTTTTT 3′.

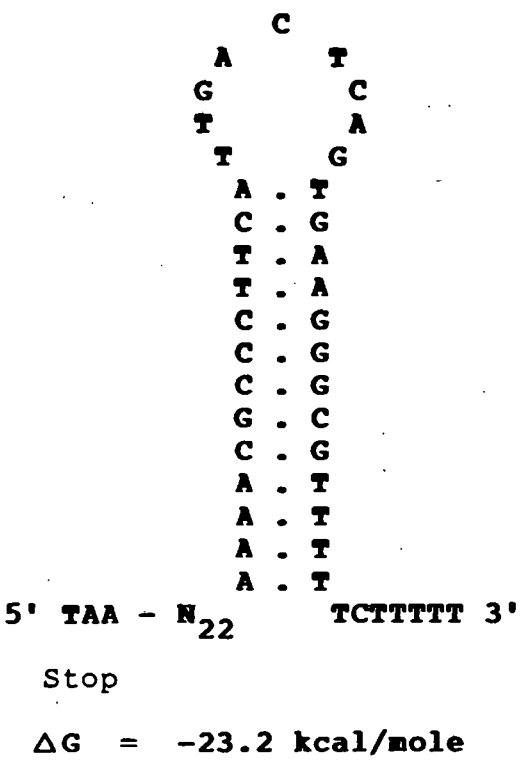

FIG. 5.6. The rho-independent transcriptional termination loop of the *scrA* structural gene from *V. alginolyticus*.

5.3.4. Sucrase amino acid sequence homology

The deduced aa sequence of the *V. alginolyticus* sucrase gene was compared to the gram-positive *B. subtilis* levansucrase, levanase and sucrase aa sequences (Steinmetz *et al.*, 1985; Martin *et al.*, 1987 b; Fouet *et al.*, 1986) and the *Saccharomyces cerevisiae* invertase encoded by the *SUC*2 gene (Taussig and Carlson, 1983). The best alignment of the *V. alginolyticus* sucrase aa sequence with that of the other related proteins showed the following percentage homologies with respect to identical aa or identical and conservatively changed residues (given in brackets): *B. subtilis* sucrase 34% (44%), *B. subtilis* levanase 23% (34%) and yeast invertase 10% (19%). No significant homology was detected between the deduced aa sequences of the *V. alginolyticus* sucrase and the *B. subtilis* levansucrase. The degree of homology between the *V. alginolyticus* and *B. subtilis* sucrase enzymes is illustrated in Figs. 5.7, 5.8 and 5.9. Fig. 5.7 shows the best alignment of the entire aa sequences, whereas Fig. 5.8 depicts the same data in the form of a matrix alignment. Fig. 5.9 indicates regions of similar secondary structure analysed by the method of Chou and Fasman (1974 a, b). Despite the relatively low percentage homology recorded for the *B. subtilis* levanase and yeast invertase when compared to the *V. alginolyticus* sucrase, three areas of striking homology were identified in all four aa sequences compared (Regions A, B and C, Fig. 5.10). The percentage of identical aa homology increased to 39, 36 and 30% for region A, 90, 80 and 60% for region B and

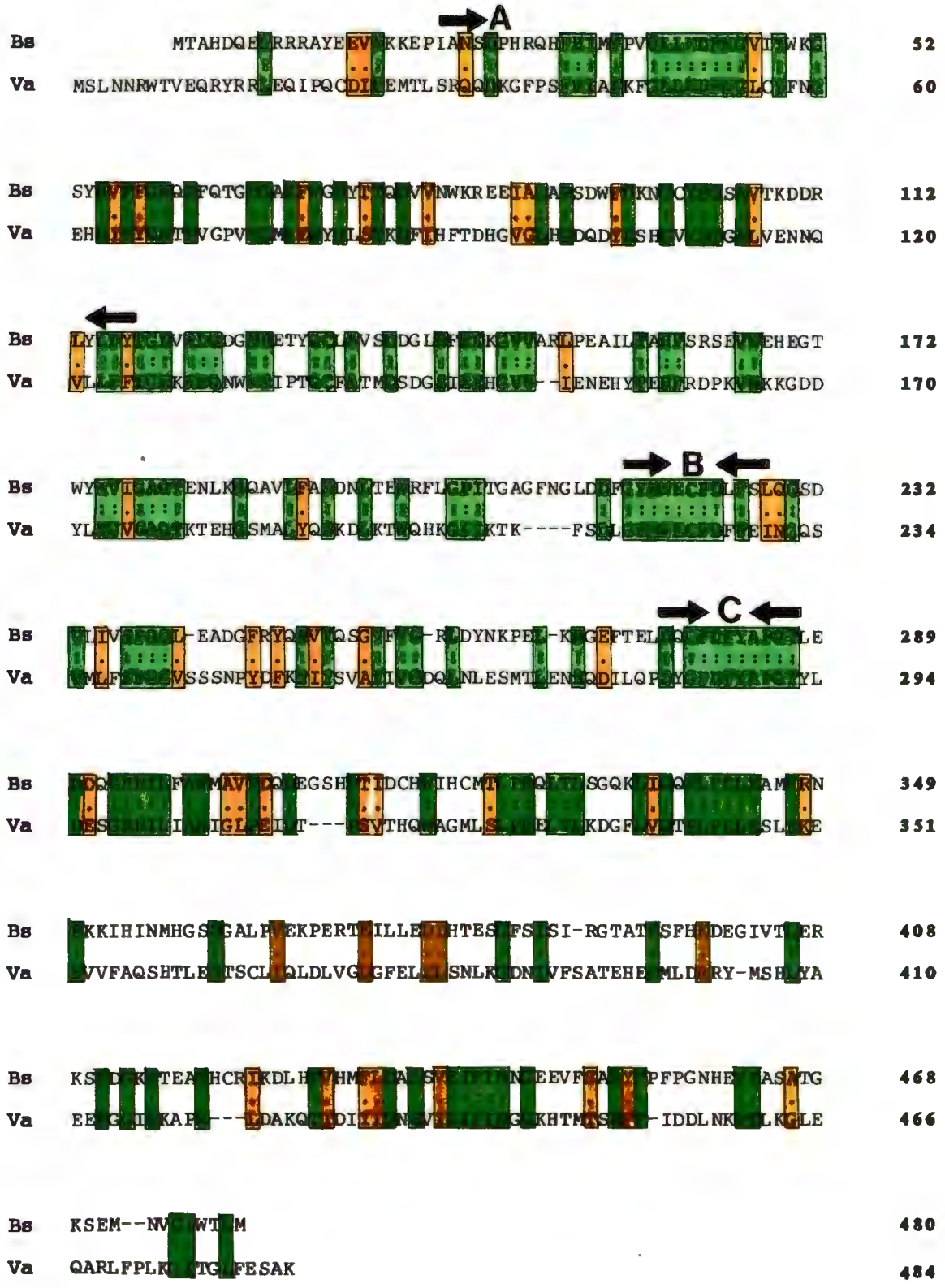

FIG. 5.7. Amino acid sequence alignment of the sucrase enzymes from *B. subtilis* (Bs) and *V. alginolyticus* (Va). The amino acids are identified by the single-letter code. Identical and conservatively changed residues are highlighted by green and orange coloured boxes, respectively. Regions A, B and C represent areas of high homology depicted in Fig. 5.10.

91, 64 and 64% for region C in comparisons of the *V. alginolyticus* sucrase with the *B. subtilis* sucrase and levanase and yeast invertase, respectively. These three regions of significant homology within the *V. alginolyticus* sucrase, the *B. subtilis* sucrase and levanase and the yeast invertase might indicate functional domains of these saccharolytic enzymes. The fact that parahydroxymercuribenzoate, a sulfhydryl group reagent, inhibits the activity of all four sucrases suggests that region B (containing the conserved cys residue) forms part of the catalytic site or at least contributes to the enzymes' active structural conformation. It should be noted that this cys is the only such residue in the *B. subtilis* levanase sequence.

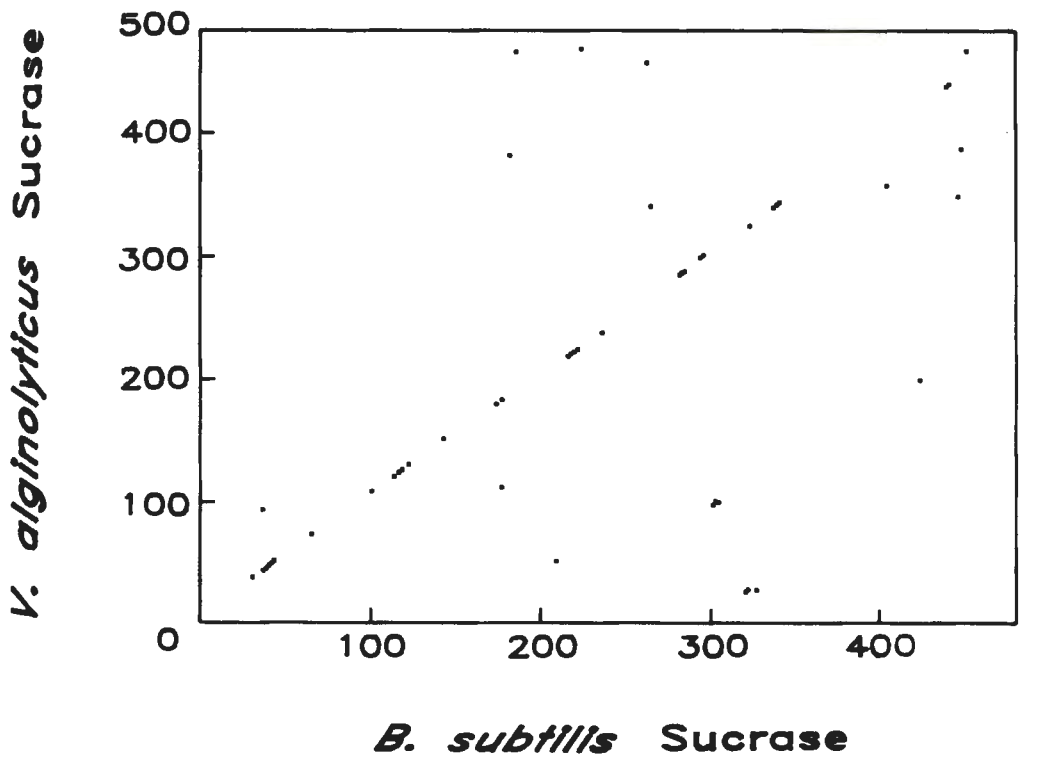

FIG. 5.8. Matrix alignment of the sucrase enzyme amino acid sequences from *V. alginolyticus* and *B. subtilis*. The window size and number of matches chosen were 6 and 4 amino acid residues, respectively. The numbering along the axes represent the residue numbers of the respective sucrase enzymes.

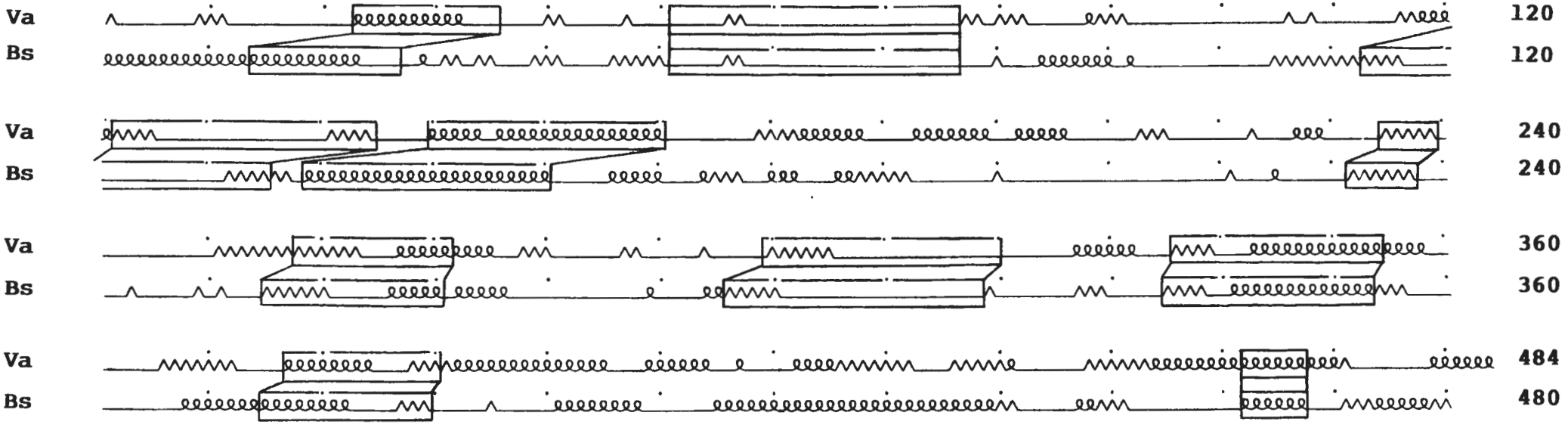

FIG. 5.9. Chou-Fasman analysis of the *V. alginolyticus* (Va) and *B. subtilis* (Bs) sucrase enzymes defined by their nucleotide sequences. Turn and random coil are indicated by continuous lines and are not distinguished. α-Helix and β-sheet are depicted by loops and zig-zags, respectively. Dots define units of 10 amino acid residues. Corresponding areas of structural similarity are boxed and connected by lines.

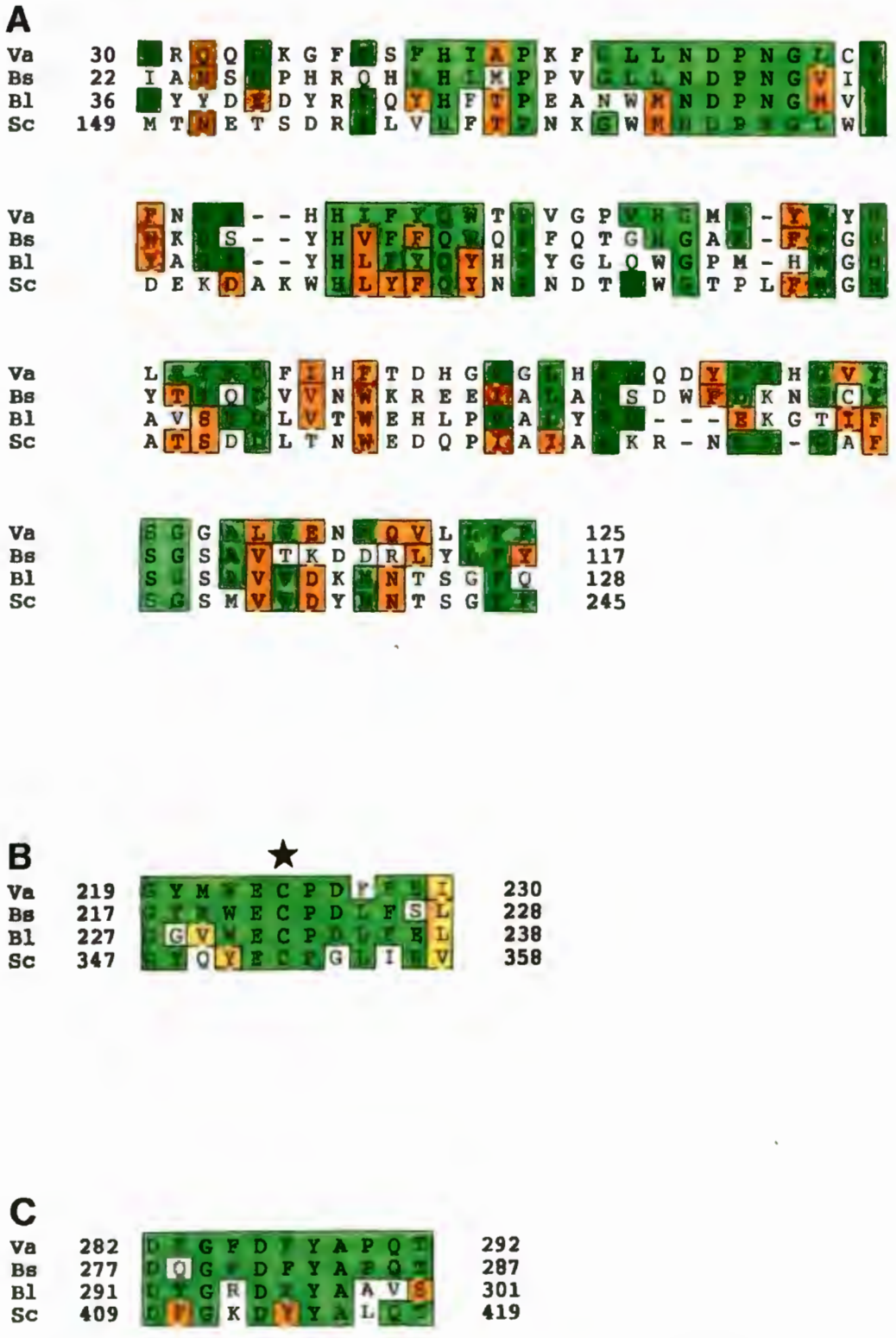

FIG. 5.10. Comparison of amino acid sequences of three regions of high homology of *V. alginolyticus* sucrase (Va) to *B. subtilis* sucrase (Bs), *B. subtilis* levanase (Bl) and *S. cerevisiae* invertase (Sc). The amino acids are identified by the single-letter code and the positions of the amino acids in the enzymes are indicated. Numbering of residues begins with the start methionine at the N-terminal end. Identical and conservatively changed residues (relative to the *V. alginolyticus* sucrase) are highlighted by green and orange coloured boxes, respectively. The conserved cys residue in region B is indicated by a star.

Although *V. alginolyticus* is a gram-negative bacterium, the homology with the sucrase enzyme of the gram-positive *B. subtilis* strain was not entirely unexpected. Research on *V. alginolyticus* has indicated other similarities between this bacterium and *Bacillus* strains. The *V. alginolyticus* and *Bacillus* strains secrete protease enzymes during the stationary growth phase (Hare *et al.*, 1981; Priest, 1977). The aa sequence of a calcium-dependent, SDS-resistant alkaline serine exoprotease 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 *Serratia marcescens* strain (Yanagida *et al.*, 1986; S. Deane personal communication).

5.4. CONCLUSION

This is the first report of the nt and aa sequence of a sucrase enzyme from a gram-negative bacterium. Amino acid homology comparisons with saccharolytic enzymes derived from *S. cerevisiae* and the gram-positive bacterium *B. subtilis* led to the identification of three possible functional domains. When the synonymic codon usage of the *V. alginolyticus* sucrase was compared to the average synonymic codon usage of 25 genes derived from the gram-negative *E. coli* strain, a similar bias was observed, which indicated a close relatedness between these two strains. Despite this result, significant aa homology was detected between the sucrase enzymes from *V. alginolyticus* and the

gram-positive *B. subtilis*. This observation provided another example of the similarity between the gram-negative *V. alginolyticus* and the gram-positive *Bacillus* strains, recognized during earlier studies on the *V. alginolyticus* exoprotease A (S. Deane, personal communication).

CHAPTER SIX

GENERAL CONCLUSION

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This study was aimed at obtaining an understanding of the sucrose utilization system from *V. alginolyticus*. Attention was given in particular to (a) the development of a recombinant *E. coli* capable of metabolizing low grade molasses, (b) the physiology of the sucrase enzyme and sucrose transport system in *V. alginolyticus* and the recombinant *E. coli* and (c) the molecular analysis of the *scrA* locus. Although most of the questions raised could be answered satisfactorily, several aspects await further investigation.

Three independent experimental approaches (osmotic shock, Tn*phoA* mutagenesis, DNA sequence determination) suggest that the heterologous sucrase enzyme is translocated across the *E. coli* cytoplasmic membrane by a mechanism not involving N-terminal modification. Although the majority of exported proteins are translocated by the 'signal-sequence-mechanism', several secreted proteins have been identified which lack amino-terminal signal sequences. Examples are the *E. coli* haemolysin and colicins and the sucrose hydrolyzing enzyme, glucosyltransferase, from *S. mutans* (Felmlee *et al.*, 1985; Pugsley and Schwartz, 1984; Robeson *et al.*, 1983). Further investigation of the mechanism of translocation of the *V. alginolyticus* sucrase enzyme in *E. coli* should contribute to the understanding of protein

movement across biological membranes mediated by a system not involving N-terminal modification.

Physiological studies on the synthesis of sucrase in *V. alginolyticus* and *E. coli* suggested the participation of a CAP, however, no region homologous to the reported CAP binding sequence was identified upstream of *scrA*. This led to the proposal that *scrA* is located on the same operon as the gene(s) encoding the transport protein(s) and that the transport and sucrose hydrolyzing functions are coordinately regulated from a common regulatory region upstream of the transport gene(s). Similarities in the responses to sucrose induction of both activities, as well as the lack of a strong promoter upstream of *scrA* seem to support this proposal. However, DNA sequence determination of the transport gene(s), which is currently in progress (G. Blatch, personal communication), is required to identify a CAP-binding region and amino acid sequencing of the first few N-terminal residues of purified sucrase enzyme is needed to verify the proposed translational start of *scrA*.

APPENDIX A

STANDARD METHODS, BUFFERS AND MEDIA

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A.1. Standard Methods

A.1.1. Large-scale isolation of *E. coli* plasmid DNA

A.1.2. Small-scale isolation of *E. coli* plasmid DNA

A.1.3. Large-scale isolation of *V. alginolyticus* chromosomal DNA

A.1.4. Plasmid purification by isopycnic CsCl-EtdBr density gradient ultracentrifugation

A.1.5. Restriction endonuclease digestion and DNA ligation reactions

A.1.6. Preparation and transformation of competent *E. coli* cells

A.1.7. DNA agarose gel electrophoresis

A.1.8. Radioactive labelling of DNA probes

A.1.9. Transfer of DNA from gels to GeneScreen membranes

A.1.10. Hybridization of DNA

A.1.11. SDS-Polyacrylamide gel electrophoresis

A.1.12. DNA sequencing

A.1.13. Gel purification of DNA endonuclease restriction fragments

A.2. Buffers and Media

A.1. Standard Methods

A.1.1. Large-scale isolation of *E. coli* plasmid DNA

Large-scale plasmid isolations were performed according to the method developed by Ish-Horowicz and Burke (1981). A 400 ml *E. coli* culture was grown overnight in LB broth (Appendix A.2) containing the appropriate selective antibiotic at 37°C and ensuring good aeration. Cells were harvested by centrifugation at 4000 x g for 10 min and resuspended in 3 ml freshly prepared Solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8.0). After 5 min at room temperature, 6 ml of freshly prepared Solution II (0.2N NaOH and 1% (w/v) SDS) were added. The suspension was mixed vigorously and placed on ice for exactly 5 min before adding 4.5 ml of precooled Solution III (5 M potassium acetate pH 4.8). After gentle shaking, the suspension was left on ice for 10 min and centrifuged at 17 000 x g for 15 min. Two volumes of 95% ethanol were added to the supernatant which was left for 5 min at room temperature. The precipitated DNA was pelleted by centrifugation at 17 000 x g for 15 min and subsequently resuspended in 4.8 ml TE buffer (Appendix A.2). The resuspended pellet was further purified by isopycnic CsCl-EtdBr ultracentrifugation (Section A.1.4).

A.1.2. Small-scale isolation of *E. coli* plasmid DNA

Small-scale plasmid isolations were carried out by the method of Ish-Horowicz and Burke (1981). A 5 ml *E. coli* culture was grown overnight in LB broth containing the appropriate selective antibiotic at 37°C with vigorous shaking. Cells were harvested from a 1.5 ml culture sample by centrifugation in an Eppendorf microfuge for 1 min. All subsequent centrifugations were performed in an Eppendorf microfuge. The pellet was drained by aspiration and resuspended in 100 µl freshly prepared Solution I (Section A.1.1. for the composition of Solutions I, II and III). The suspension was kept for 5 min at room temperature, before being placed on ice for 1 min. Following the addition of 200 µl of cool freshly prepared Solution II, the mixture was briefly vortexed and kept on ice for 5 min. Ice cold Solution III (150 µl) was added, the suspension was briefly vortexed and placed on ice for another 5 min. The sample was centrifuged for 5 min, the supernatant transferred into a fresh tube and two volumes of 95% ethanol added. After mixing the suspension thoroughly, it was kept at room temperature for 5 min, centrifuged for 10 min and dried. The dry pellet was resuspended in 150 µl TE buffer. Sodium acetate (3 M, 10 µl) and 300 µl of 95% ethanol was added. The sample was briefly vortexed and kept at -20°C for 30 min. The DNA was pelleted by centrifugation for 20 min, the supernatant was decanted and the pellet was dried before resuspending in 50 µl TE buffer.

A.1.3. Large-scale isolation of *V. alginolyticus* chromosomal DNA

Chromosomal DNA isolations from *V. alginolyticus* were carried out by the method of Marmur (1961). A 200 ml *V. alginolyticus* culture was grown overnight at 30°C in LB broth (Appendix A.2) supplemented with 0.4 M NaCl. The cells were harvested by centrifugation at 4000 x g for 10 min and washed with 50 ml saline-EDTA (0.15 M NaCl and 0.1 M EDTA). After resuspending the pellet in 25 ml saline-EDTA, the cells were lysed by the addition of 10 mg lysozyme followed by incubation at 37°C for 30-60 min with occasional shaking. After lysis was complete, 2.0 ml sodium lauryl sulfate (25%) was added and the mixture was kept at 60°C for 10 min before cooling to room temperature. Sodium perchlorate (5 M) was added to a final concentration of 1 M to the lysed suspension and the mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) for 30 min. The resulting emulsion was separated into 3 layers by a 5-min centrifugation at 7000 x g. The upper aqueous phase, containing the nucleic acids, was carefully removed, two volumes of 95% ethanol was added and the precipitated nucleic acids were pelleted by centrifugation at 17 000 x g for 15 min. The pellet was resuspended in 15 ml saline-citrate (0.15 M NaCl and 0.015 M trisodium citrate pH 7.0), shaken as before with an equal volume of chloroform-isoamyl alcohol for 15 min, centrifuged and the upper phase removed. The mixture was then deproteinized repeatedly with chloroform-isoamyl alcohol until very little protein was visible at the interface. DNA in the aqueous phase obtained

after the last in the series of deproteinizations was precipitated with two volumes of 95% ethanol and resuspended in half the volume of saline-citrate. RNase was added to a final concentration of 50 μg/ml and the mixture was incubated for 30 min at 37°C. The digest was again subjected to a series of deproteinizations until there was little denatured protein visible at the interface after centrifugation. The DNA in the upper phase was again precipitated with two volumes of 95% ethanol, the pellet was drained, washed in 70% ethanol, dried and dissolved in 2-3 ml TE buffer (Appendix A.2). The nucleic acid concentration was determined spectrophotometrically. One absorbance unit (260 nm) was considered equivalent to 50 μg DNA/ml.

A.1.4. Plasmid purification by isopycnic CsCl-EtdBr density gradient ultracentrifugation

Crude plasmid preparations were purified by isopycnic CsCl-EtdBr density gradient ultracentrifugation as outlined by Maniatis *et al.*, (1982). To crude plasmid preparations, resuspended in TE buffer, CsCl (final concentration 1 g/ml) and EtdBr (final concentration 400 μg/ml) were added. The preparation was clarified by centrifugation at 27 000 x g for 15 min and the refractive index of the supernatant was adjusted to 1.398. The sample was sealed in a Beckman Quickseal ultracentrifuge tube and centrifuged for 14 hours at 340 000 x g and 15°C in a Beckman VTi65 rotor. The plasmid bands were visualized under long wave UV light (350 nm), and the lower covalently closed circular plasmid band was removed (approximately 700 μl) with a syringe fitted

with a wide bore needle. The EtdBr was removed by adding two volumes salt saturated isopropanol, shaking the sample vigorously and allowing the phases to separate. EtdBr in the upper isopropanol phase was carefully removed by aspiration. Isopropanol extractions were repeated until no traces of EtdBr were visible. The DNA was precipitated by adding two volumes sterile water and two final volumes 95% ethanol before keeping the sample at -20^{o}C for 3 h. The DNA was pelleted by repeated centrifugation for 10 min at room temperature in an Eppendorf microfuge. The pellet was dried and resuspended in 200 μl TE buffer (Appendix A.2), and the concentration of nucleic acids was determined spectrophotometrically. One absorbance unit (260 nm) was considered equivalent to 50 μg/ml.

A.1.5. Restriction endonuclease digestion and DNA ligation reactions

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

DNA ligation reactions were carried out at room temperature for 4-14 h in a total volume of 20 μl ligation buffer (Appendix A.2) and in the presence of 1 and 2 units

of T4 DNA ligase (Boehringer Mannheim)/μg DNA for sticky and blunt end ligations respectively. The total amount of DNA present in vector-insert and closing-circle ligations was 10 to 15 pmole and 1 pmole, respectively. Vector and insert DNA was added to the ligation reactions at a molar ratio of 1:2.

A.1.6. Preparation and transformation of competent *E. coli* cells

Competent *E. coli* cells were prepared by a modification of the methods described by Cohen *et al.*, (1972) and Dagert and Ehrlich (1979). A 10 ml *E. coli* culture was grown overnight in LB broth (Appendix A.2) at 37^{o}C. An inoculum of this culture (2 ml) was added to 100 ml of prewarmed LB broth and incubated at 37^{o}C with vigorous shaking until an OD_{600} of 0.2 was reached. The culture was chilled on ice and kept at 4^{o}C for all subsequent steps. The cells were harvested by centrifugation at 2000 x g for 5 min, washed with 10 ml ice cold 0.1 M $MgCl_2$ and recentrifuged at 2000 x g for 5 min. The pellet was subsequently resuspended in 10 ml ice cold 0.1 M $CaCl_2$ and kept on ice. After 30 min, the cells were harvested by centrifugation at 2000 x g for 5 min, resuspended in 2 ml ice cold $CaCl_2$ and allowed to age for approximately 3 h.

Plasmid DNA (50 to 200 ng), resuspended in TE buffer, was added to 100 μl of competent, aged *E. coli* cells. The mixture was kept on ice for 10 min, followed by a heat shock treatment at 42^{o}C for 5 min, after which the cells were returned to ice for a further 2 min. After adding 400 μl LB

broth to each transformation, the cells were incubated at 37°C for 45 to 60 min to allow expression of selective antibiotic resistance markers encoded by the transferred DNA. A sample of 100 µl was spread onto agar plates containing the appropriate selective antibiotic. The following controls were routinely included: competent cells with no DNA added and uncut plasmid pBR322 or Bluescript SK to monitor the transformation efficiency. Approximately 5000 colonies/ng control plasmid transformed were routinely obtained.

A.1.7. DNA agarose gel electrophoresis

Electrophoresis of DNA was carried out using horizontal gel systems with TBE or Tris-acetate buffer (Appendix A.2). Agarose concentrations varied between 0.8 and 1.2%, depending on the size of DNA fragments to be resolved. DNA samples were mixed with a quarter volume of DNA sample loading buffer (Appendix A.2). DNA agarose gels were stained with 0.8 µg EtdBr/ml electrophoresis buffer and the DNA visualized using a 254 nm wavelength Transilluminator (Chromato-Vue Model TS-15, UV Products Inc., San Gabriel, CA, USA). Agarose gels were photographed using a Polaroid CU-5 Land camera and Polaroid 667 film. 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 endonuclease digestion of λ DNA with *Pst*I, *Hin*dIII or *Eco*RI.

A.1.8. Radioactive labelling of DNA probes

Radioactively labelled DNA probes were prepared by nick-translation using kit PB.5025 supplied by Amersham according to the manufacturer's instructions. Unincorporated ^{32}P-labelled nt were separated from the labelled probe using a Sephadex G-50 spin column as described by Maniatis *et al.*, (1982).

A.1.9. Transfer of DNA from gels to GeneScreen membranes

DNA fragments were resolved by Tris-acetate-buffered agarose gel electrophoresis and were transferred to GeneScreen hybridization transfer membranes (New England Nuclear Corp., Boston, MA, USA) by the method of Smith and Summers (1980). Gels were washed in two volumes 0.25 M HCl for 15 min, rinsed in distilled water, washed twice in two volumes 0.5 M NaOH, 1.5 M NaCl for 15 min and were neutralized by washing them twice in two volumes 1 M ammonium acetate, 0.02 M NaOH for 30 min. All washing steps were performed with gentle agitation. GeneScreen membranes, cut to the size of the gels, were soaked in neutralizing buffer and placed onto the gel resting on a flat, clean surface. Three sheets of Whatman 3MM filter paper, soaked in neutralizing buffer, were placed onto the GeneScreen membrane. A 4 cm layer of dry paper towel was pressed down on the filter paper by a glass plate and a 1 kg weight. DNA transfer from the gel onto the membrane was allowed to take place for 2 h, after which the GeneScreen membrane was briefly washed in 2 x SSC (Appendix A.2), air dried and baked in a vacuum oven for 2 h at 80^{o}C.

A.1.10. Hybridization of DNA

Radioactively labelled DNA probes were hybridized to DNA immobilized onto GeneScreen membranes by soaking the baked membrane in 6 x SSC (Appendix A.2) for 2 min before transferring it into a plastic bag containing 60 ml prehybridization fluid (6 x SSC, 0.5% SDS, 5 x Denhardt's solution, 100 μg/ml denatured Salmon sperm DNA) (Appendix A.2). Prehybridization was carried out at 68^{o}C with continuous gentle agitation. After 4 h the prehybridization fluid was replaced by 15 ml hybridization solution (6 x SSC, 10 mM EDTA, 5 x Denhardt's solution, 0.5% SDS, 100 μg/ml denatured Salmon sperm DNA and 100 μl probe which was boiled for 5 min). Hybridization was carried out for 18 h at 68^{o}C with continuous gentle agitation. Filters were subsequently washed once in 2 x SSC, 0.1% SDS at 60^{o}C for 15 min and four times in 0.2 x SSC, 0.1% SDS for 15 min at 60 ^{o}C.

The membrane, sealed into a plastic bag, was exposed to Kodak XAR-5 autoradiographic film for 7-14 days at -70^{o}C.

A.1.11. SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gels (15%) were prepared according to the method of Laemmli (1970) and O'Farrell (1975) using a Hoeffer SE600 vertical slab gel apparatus. The resolving gel was prepared according to the table below, degassed and poured. Water was layered on the gel to ensure the formation of a sharp interface. After the resolving gel had set (30 min at room temperature) the water was removed and the stacking gel, prepared according to the table below, was

cast. The comb, presoaked in ammonium persulphate, was inserted and left until the gel had set.

Samples were prepared in SDS sample buffer (4 µl 0.125 M HCl, 2.5 µl 10% SDS, 2 µl glycerol, 1.5 µl distilled water) boiled for 2 min and 10 µl of 0.1% Bromophenol Blue solution was added, before being loaded onto the gel. Samples were run at 150 V through the stacking gel and at 25 mA constant current through the resolving gel. After electrophoresis was complete, the gels were stained for 1 h in 0.05% Coomasie Brilliant Blue in destain (90 ml acetic acid, 810 ml distilled water, 300 ml methanol). The gels were then destained and dried.

SDS-PAGE buffers

Acrylamide-bis-acrylamide stock solution

Acrylamide	29.2 g
Bis-acrylamide	0.8 g

These components were dissolved in distilled water and made up to 100 ml. Active charcoal (5 g) was added to the solution and the solution stirred for 8 h before filtering it through Whatman paper No.1.

Running gel buffer

Tris	1.5 M
SDS	0.4% (w/v)
pH 8.8	

Reservoir buffer (10 x)

Tris	0.067 M
Glycine	0.238 M
SDS	1.0% (w/v)

Stacking gel buffer

Tris	0.5 M
SDS	0.4% (w/v)
pH 6.8	

Preparation of 15% acrylamide gels

Solution	Running gel	Stacking gel
Acrylamide stock solution	18.0 ml	3.0 ml
Running gel buffer	8.2 ml	-
Stacking gel buffer	-	3.0 ml
Distilled water	7.65 ml	6.0 ml
Ammonium persulphate (10%)	160.0 µl	64.0 µl
TEMED	18.0 µl	13.0 µl

A.1.12. DNA sequencing

Sequencing templates, prepared by isopycnic CsCl-EtdBr density gradient ultracentrifugation (Section A.1.4), were further purified by an ammonium acetate precipitation. To a DNA preparation, resuspended in TE buffer, an equal volume of 8 M ammonium acetate and two volumes of absolute ethanol were added. The precipitation was allowed to proceed for 16 h at -20°C after which the precipitate was recovered by centrifugation in an Eppendorf microfuge for 1 h and dried *in vacuo* for a further h. The pellet was resuspended in 0.1 x TE buffer (Appendix A.2) and the DNA concentration determined spectrophotometrically as described in Section A.1.4.

The double stranded DNA templates were denatured and annealed to primers according to the following procedure. Supercoiled DNA (2-4 μg) was diluted to a final volume of 20 μl in distilled water before alkaline denaturation in 0.2 M NaOH for 5 min at 22°C. This mixture was subsequently subjected to ethanol precipitation by the addition of 5 μl of 3 M sodium acetate, 25 μl of distilled water and 150 μl of absolute ethanol. The precipitation was briefly chilled at -70°C, the precipitate recovered by centrifugation in an Eppendorf microfuge for 20 min at 4°C and residual salts were removed by a wash with 200 μl of 70% ethanol. The pellet was subsequently dried and resuspended in 10 μl sequencing buffer (40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) to which 12 ng of primer was added. The primer was

allowed to anneal to the template for 15 min at 40°C immediately prior to sequencing.

DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method developed by Sanger *et al.*, (1977) according to the protocol outlined by Tabor and Richardson (1987) using the Sequenase DNA Sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The manufacturer's instructions were followed throughout. The DNA chains were radiolabelled with [α-^{35}S]dATP (specific activity 1200 Ci/mmol, Amersham) and extended using T7 DNA polymerase supplied by Boehringer Mannheim. Sequencing ladders were resolved on standard 6% acrylamide urea sequencing gels of a thickness of 0.2 mm.

After electrophoresis, gels were placed under Kodak XAR-5 X-ray film in an X-ray cassette and exposed for 24 h to 3 days.

A.1.13. Gel purification of DNA endonuclease restriction fragments

Gel purification of DNA endonuclease restriction fragments was carried out by the method of Struhl (1985). DNA was digested with restriction endonucleases as described in Section A.1.5. The DNA restriction fragments were resolved by electrophoresis in 40 mM Tris-acetate (pH 8.1)-buffered 0.8% SeaPlaque (Marine Colloids, Rockland, ME) low melting point agarose gels. The gel was stained in sterile, distilled water containing 0.05 μg/ml EtdBr for 10 min. The restriction bands were visualized under long wave UV light (350 nm) and were excised in the smallest possible volume

with a clean blade. Ligation reactions using gel purified DNA fragments were carried out as described in Section A.1.5. Gel slices containing DNA were heated to 70°C for 5 to 10 min to allow transfer and mixing of the required volume. The DNA sample was added to the ligation mixture last. Prior to transforming competent *E. coli* cells (Section A.1.6), the solidified ligation mixture was reheated as described above and the required amount removed and diluted in twice the volume of 0.1 M $CaCl_2$ to prevent the ligation mixture from resolidifying.

A.2. Buffers and Media

CSH MM salts (5 x)

K_2HPO_4	52.5 g
KH_2PO_4	22.5 g
$(NH_4)_2SO_4$	5 g
Sodium citrate	2.5 g
Distilled water	1000 ml

Denatured salmon sperm DNA (Maniatis *et al.*, 1982)
Lyophilized salmon sperm DNA was dissolved in distilled water (10 mg/ml) and sheared by passing the solution several times through an 18-gauge hypodermic needle. The DNA was denatured by boiling for 10 min, immediately placed on ice and stored in 1 ml aliquots at -20°C. The DNA was boiled for 5 min and placed on ice prior to use.

Denhardt's solution (10 x) (Maniatis *et al.*, 1982)

Ficoll	1% (w/v)
Polyvinylpyrrolidone-40	1% (w/v)
BSA (Fraction V, Sigma)	1% (w/v)

Filter sterilized and stored at -20°C.

Dinitrosalicylic acid reagent (DNS) (Miller, 1959)

3.5 Dinitrosalicylic acid	10.6 g
NaOH	19.8 g

After dissolving the above in 1416 ml distilled water the following was added:

Na K Tartrate	306 g
Phenol	7.6 ml
Na meta bisulfite	8.3 g

A 3 ml sample was titrated with 0.1N HCl using phenolphthalein as pH indicator. Neutralization required 5 to 6 ml HCl.

DNA sample loading buffer

Bromophenol blue	0.25% (w/v)
Glycerol	50% (v/v)
EDTA	100 mM

Exobuffer (pH 8.0) (Henikoff, 1984)

$MgCl_2$	0.66 mM
Tris-HCl	66 mM

H agar

Tryptone	10 g
NaCl	8 g
Agar	12 g
Distilled water	1000 ml

H top agar

Tryptone	10 g
NaCl	8 g
Agar	8 g
Distilled water	1000 ml

Klenow buffer (pH 8.0) (Henikoff, 1984)

Tris-HCl	20 mM
$MgCl_2$	7 mM

LB agar (Maniatis *et al.*, 1982)

LB broth containing 1.5% agar.

LB broth (Maniatis *et al.*, 1982)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

For the propagation of *V. alginolyticus* in LB broth, an additional 23.4 g of NaCl was added.

Ligation buffer (10 x) (pH 7.6)

Tris-HCl	0.5 M
$MgCl_2$	0.1 M
Dithiothreitol	0.1 M
Adenosine triphosphate	10 mM

MacConkey agar

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

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

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 after autoclaving with the exception of NaCl and vitamin-free Casamino acids, which were omitted.

MM broth

5 x CSH MM salts	200 ml
Distilled water	800 ml

Autoclaved separately

The following sterilized solutions were added

Carbon source (20%, w/v)	10 ml
$MgSO_4$ (20%, w/v)	1 ml
Vitamin B1 (0.5%, w/v)	1 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

For the propagation of *V. alginolyticus* in MM broth, the addition of vitamin-free Casamino acids was omitted and the final concentration of NaCl was increased to 2.34% (w/v).

Peptone/skim agar

Peptone (Merck, Darmstadt)	25 g
NaCl	70 g
Agar	22.5 g
Tris (0.1 M, pH 7.6)	900 ml
Autoclaved separately	
Skim milk	10 g
Distilled water	100 ml
Autoclaved separately	

Peptone/skim agar was used for the propagation of *V. alginolyticus* stock cultures.

Phosphate buffers

Phosphate buffers were prepared as described by Williams and Chase (1968).

PolI buffer (10 x) (pH 7.5-7.9)

Tris-HCl	0.1 M
$MgCl_2$	0.1 M
NaCl	0.5 M
β-mercaptoethanol	0.7 M
Dithiothreitol	10 mM

SI buffer (10 x) (Henikoff, 1984)

Potassium acetate	0.3 M
NaCl	2.5 M
Glycerol	50%
$ZnSO_4$	10 mM

SI stop buffer (pH 8.0) (Henikoff, 1984)

Tris-OH	0.5 M
EDTA	0.125 M

SSC buffer (pH 7.0)

NaCl	0.15 M
Sodium citrate	0.015 M

Starch-iodine solution (Sykes and Nordstrom, 1972)

Starch	0.2 g
Distilled water	100 ml

The solution was boiled for 2-3 min until nearly clear and 0.15 ml of the iodine solution was added.

Iodine solution

I_2	0.08 M
KI	3.2 M
Distilled water	10 ml

TBE buffer (pH 8.0)

Tris	89 mM
Boric acid	89 mM
EDTA	2.5 mM

TE buffer (pH 8.0)

Tris-HCl	10 mM
EDTA	1 mM

TE-equilibrated phenol

To 100 ml phenol, melted at 68°C, 8-hydroxyquinoline was added to a final concentration of 0.1%. The mixture was equilibrated with two volumes of 1.0 M Tris pH 8.0 and two volumes of 0.1 M Tris pH 8.0-0.2% β-mercaptoethanol by mixing the phases thoroughly and allowing them to separate. The equilibrated phenol was stored under 0.1 M TE buffer pH 8.0 at -20°C in the dark.

Tokuda MM (Tokuda *et al.*, 1981)

NaCl	86 mM
KCl	10 mM
K_2HPO_4	2 mM
$(NH_4)_2SO_4$	15 mM
Tris-HCl (pH 7.5)	50 mM

Autoclaved separately

The following sterilized solutions were added (per liter)

$MgSO_4$ (0.5 M)	10 ml
$FeSO_4$ (1 mM)	1 ml
Carbon source (50%, w/v)	10 ml
Vitamin B1 (0.5%, w/v)	1 ml
L-Amino acids (20 mg/ml)	1 ml
Vitamin-free Casamino acids (Difco Laboratories) (25%, w/v)	10 ml

Tris-acetate buffer (pH 8.0)

Tris	40 mM
Glacial acetic acid	20 mM
EDTA	2 mM

For tris-acetate buffered electrophoresis using SeaPlaque low melting point agarose, the addition of EDTA was omitted.

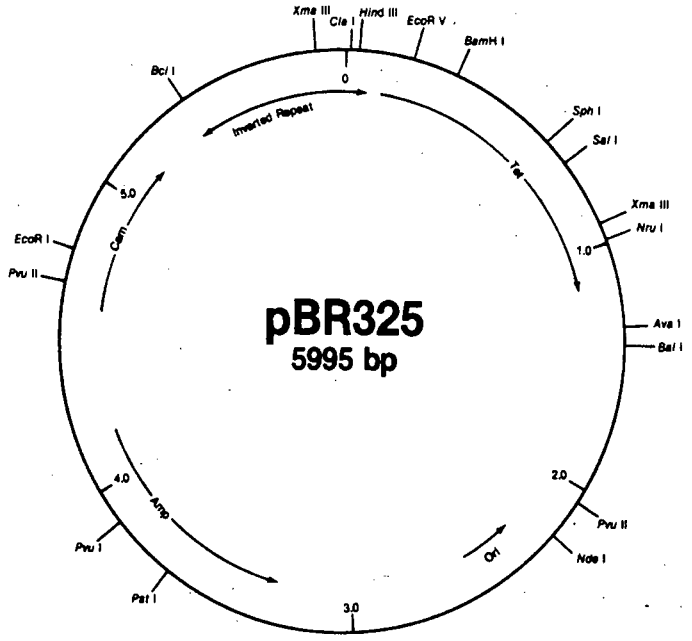
APPENDIX B

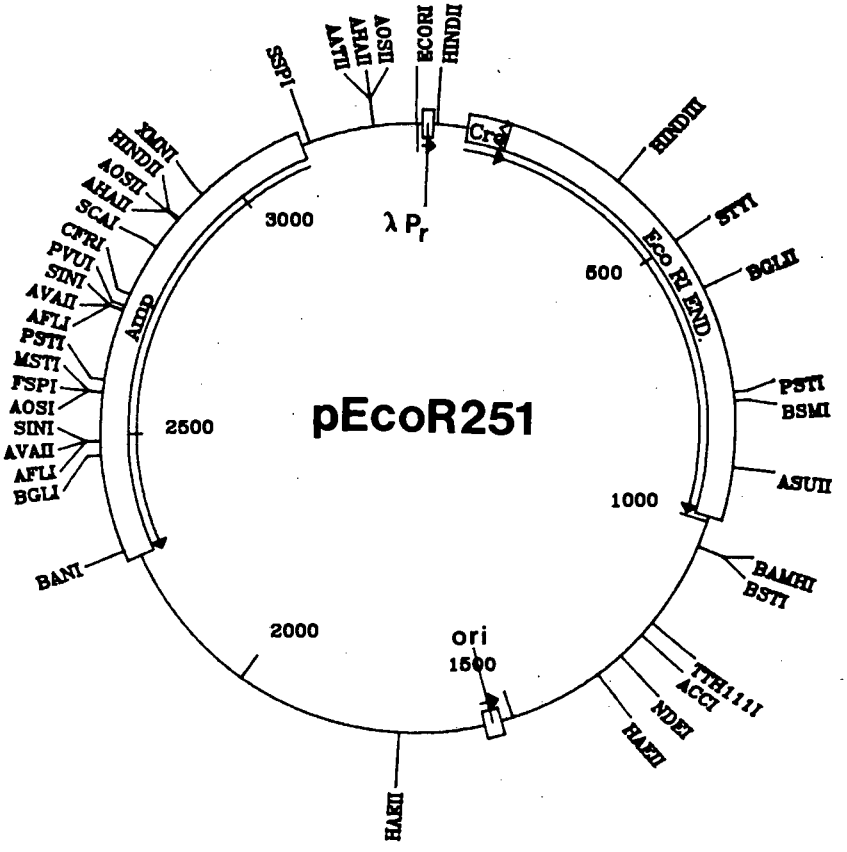
CLONING VECTOR AND TRANSPOSON RESTRICTION MAPS

APPENDIX B

CLONING VECTOR AND TRANSPOSON RESTRICTION MAPS

pBR325	Bolivar (1978)
pEcoR251	Zabeau and Stanley (1982), Remaut *et al.*, (1983)
Bluescript SK	Stratagene, San Diego
M13mp18	Messing (1983)
Tn*phoA*	Manoil and Beckwith (1985), Gutierrez *et al.*, (1987)


pBR325
5995 bp
Xma III
Cla I
Hind III
EcoR V
BamH I
Sph I
Sal I
Xma III
Nru I
Ava I
Bal I
Pvu II
Nde I
Pst I
Pvu I
EcoR I
Pvu II
Bcl I
Inverted Repeat
Tet
Cam
Amp
Ori
0
1.0
2.0
3.0
4.0
5.0


pEcoR251
λ Pr
Cro
Eco RI END
Amp
ori
1500
3000
500
1000
2000
2500
SSPI
ECORI
HINDII
HINDIII
STYI
BGLII
PSTI
BSMI
ASUII
BAMHI
BSTI
TTH111I
ACCI
NDEI
HAEII
HAEIII
BANI
BGLI
AFLI
AVAII
SINI
AOSI
FSPI
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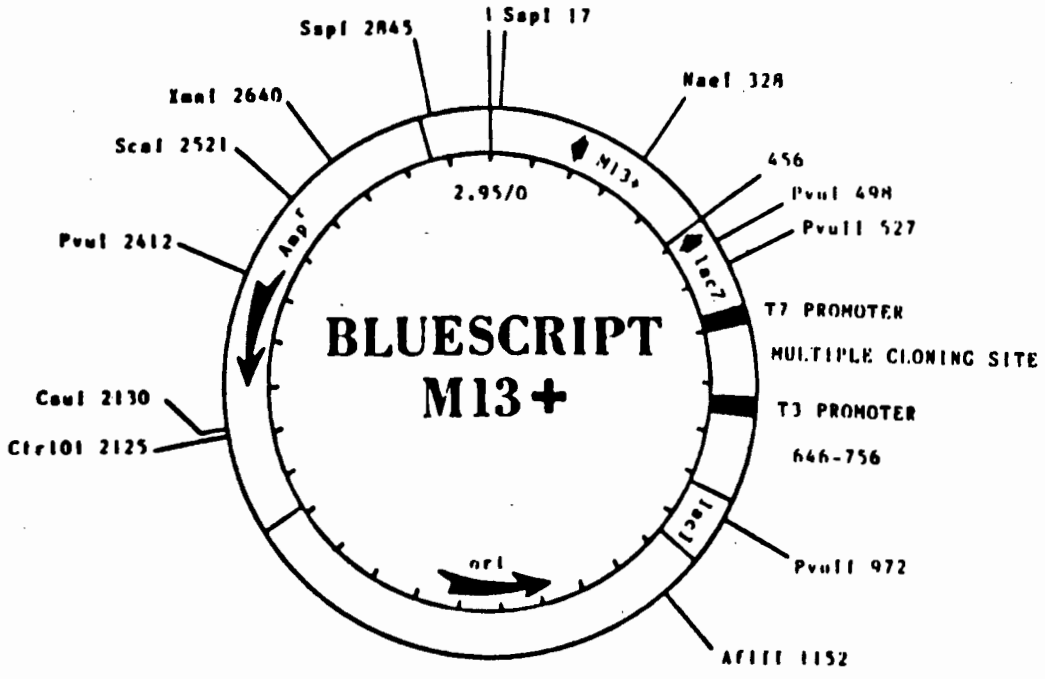

SK POLYLINKER

```
                    HincII
      ApaI          AccI                 EcoRV
KpnI  DraII    XhoI  SalI     ClaI  HindIII     EcoRI PstI
GGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG
CCATGGCCCGGGGGGGAGCTCCAGCTGCCATAGCTATTCGAACTATAGCTTAAGGACGTC

                          EagI     SacII
SmaI  BamHI SpeI  XbaI  NotI    BstXI       SacI
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GGGCCCCCTAGGTGATCAAGATCTCGCCGGCGGTGGCGCCACCTCG
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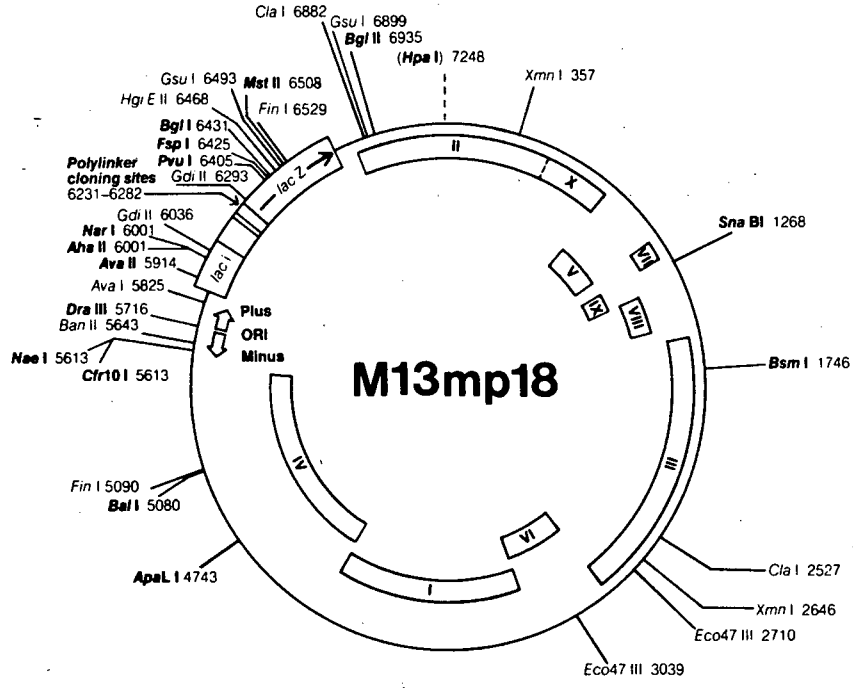
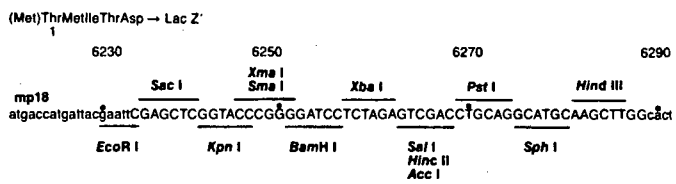
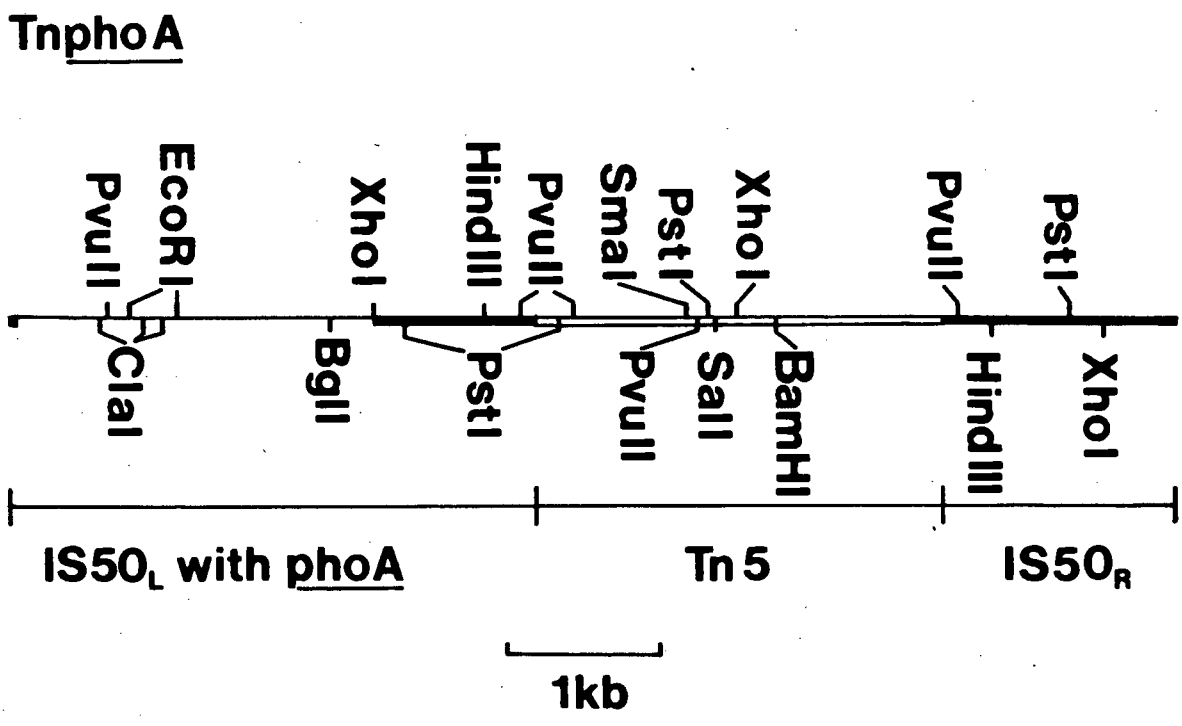


Scale:
240 base pairs/cm.





APPENDIX C

LITERATURE CITED

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