RESULTS

4.1 INTRODUCTION:

In the previous chapter, the isolation and phenotypic characterization of a mutant GJ1055, in which proU regulation was affected in trans was described. GJ1055 was shown to carry a mutation dpeA, allelic to hupB, the gene coding for one of the two subunits of the nucleoid protein HU. The effect of the dpeA mutation on proU was shown to be mediated through the P2R mechanism of regulation. It was noted that, in the chromosomal background of the original mutant GJ1055, there was also a pronounced reduction in lac expression from plasmid pHYD275 (carrying the P1 promoter), and that this could not be accounted for solely by the dpeA mutation in this strain. The possibility was therefore considered that GJ1055 harbors a second mutation affecting P1 promoter expression, and this mutation was tentatively named as dpeB. The mapping of dpeB, identification of its gene product and other studies related to dpeB are described in this chapter.

4.2 OBTAINING Tn10 INSERTION LINKED TO dpeB:

In a P1 transduction with GJ1064 (carrying a Tn10dKan insertion 67% linked to dpeA+) as donor and GJ1055/pHYD275 (dpeA dpeB) as recipient, 100% of the Kanr transductants continued to be Lac- (although 67% would have been expected to become dpeA+). This observation was demonstrative of the presence of a second mutation dpeB in GJ1055 that affects expression from the P1 promoter on pHYD275. This experiment also indicated that dpeB is unlinked to dpeA, and probably acts independently of dpeA to affect proU promoter P1 expression.

In order to obtain a transposon insertion marker close to dpeB, random transpositions of Tn10dKan were generated in GJ134 (dpeB+) with the aid of A1105 as described in Chapter 2. A P1 lysate prepared on a population of these Kanr cells was then used in a transduction into GJ1055/pHYD275 (dpeA dpeB) as recipient, with a selection for Kanr transductants on MacConkey plates supplemented with uracil, Tp and NaCl (0.15 M). On this medium, GJ134/pHYD275 (dpeB+) is Lac+ and
GJ1055/pHYD275 (dpeB) is Lac\(^{+}\), and the concentration of NaCl is sufficient as not to significantly affect the efficiency of plating of the transductants. Approximately one percent of the Kan\(^{R}\) transductants was Lac\(^{+}\), which most likely represented the clones in which the dpeB allele of GJ1055/pHYD275 had been replaced by dpeB\(^{+}\) in linked transduction with Kan\(^{R}\) insertion.

The degree of linkage between Kan\(^{R}\) and dpeB\(^{+}\) in each of the latter clones was determined in transduction experiments in which a P1 lysate grown on each was individually used to transduce GJ1055/pHYD275 again to Kan\(^{R}\), and the resultant transductants were screened for loss of the mutant (dpeB) phenotype (Table 4.1). In one case (#9), 76% (475/625) of Kan\(^{R}\) colonies had become Lac\(^{+}\) indicating that the Tn10dKan insertion in this clone was 76% cotransducible with dpeB\(^{+}\) (Table 4.1). A P1 lysate prepared on one of the Kan\(^{R}\) transductants (from this cross) that retained the dpeB mutation was then used to construct an isogenic dpeB derivative of GJ134/pHYD275, that was designated GJ1074.

### 4.3 EFFECT OF dpeB MUTATION ON THE VARIOUS MODES OF proU REGULATION:

A Tp\(^{S}\) derivative of GJ1074 (dpeB), GJ1075, was obtained following growth in antibiotic-free medium and screening for Tp\(^{S}\) clones. To monitor the effect of dpeB on the various modes of proU regulation, expression from each of the plasmids pHYD275, pHYD284, pHYD283 and pHYD272 were studied as a function of medium osmolarity in the corresponding plasmid-transformants of the dpeB strain GJ1075, and the values obtained were compared to those of the corresponding wild-type (GJ134) derivatives. The results, presented in Figure 4.1, clearly indicate that the dpeB mutation causes a reduction in expression from promoter P1 of proU (present in pHYD275). However, osmotic induction of proU P1 seems to be unaffected by the dpeB mutation, as is evident from the observation that the residual expression from pHYD275 in the dpeB mutants continues to show a five-fold induction (see inset to Fig. 4.1). Expression from plasmid pHYD284 (possessing P2R) was not affected in the dpeB strain, indicating that P2R mechanism of regulation is not affected by dpeB.
TABLE 4.1. Linkage of different Tn10dKan isolates to \textit{dpeB}^{+} locus in P1 transduction$^a$.

<table>
<thead>
<tr>
<th>Tn10dKan isolate no.</th>
<th>%Cotransduction frequency (No. of Kan\textsuperscript{r} \textit{dpeB}^{+}/No. of Kan\textsuperscript{r})</th>
</tr>
</thead>
<tbody>
<tr>
<td>#2</td>
<td>52 (26/50)</td>
</tr>
<tr>
<td>#4</td>
<td>12 (9/72)</td>
</tr>
<tr>
<td>#9</td>
<td>76 (42/55)</td>
</tr>
<tr>
<td>#13</td>
<td>6 (4/65)</td>
</tr>
<tr>
<td>#14</td>
<td>37 (18/49)</td>
</tr>
<tr>
<td>#21</td>
<td>49 (27/55)</td>
</tr>
</tbody>
</table>

$^a$Donors carried the Tn10dKan linked to \textit{dpeB}^{+}. The genotype of the recipient (GJ1055/pHYD275) was \textit{proU-lac dpeA dpeB}. 
Fig. 4.1. Effect of dpeB mutation on the various modes of proU regulation. Derivatives of GJ134 (wild-type, □) and GJ1075 (dpeB, O) carrying the various Tp plasmids (as mentioned for each panel), were grown to mid-log phase in K medium supplemented with various concentrations of NaCl as indicated. Panels: A, pHYD275 (P1R); B, pHYD284 (P2R); C, pHYD283 (P1R+P2R); and D, pHYD272 (P1R+P2R+NRE). Inset to panel A shows enzyme activity values for pHYD275 derivative of GJ1075 replotted on an expanded ordinate scale.
One would expect the \textit{dpeB} effect on promoter P1 to be manifest even in its native context, that is when present along with P2R and P2R+NRE. Unexpectedly, however, expression from plasmid pHYD272 (possessing P1R+P2R+NRE) and also pHYD283 (possessing P1R+P2R) were not affected by the \textit{dpeB} mutation. The reason for unaltered expression from pHYD272 and pHYD283 in the \textit{dpeB} background in this experiment is not completely understood, but some possibilities are discussed below.

Based on the results above, it was concluded that the \textit{dpeB} mutation specifically affects expression from the promoter P1 and does not interfere with P2R or NRE-mediated regulation.

\section*{4.4 Mapping of \textit{dpeB} Locus:}

To facilitate mapping of the \textit{dpeB} locus, the approximate map position of the Tn10dKan insertion that was 76\% linked to \textit{dpeB}, was first determined in a series of conjugation experiments. Based on the data obtained and described below the Tn10dKan insertion was placed near 59 min on the \textit{E. coli} chromosome and has accordingly been designated \textit{zfl-2102::Tn10dKan}.

\subsection*{4.4.1 Conjugation Experiments:}

\subsubsection*{4.4.1.1 Conjugation with a set of Tet\textsuperscript{r} Hfr Strains:}

As had been done earlier in the experiments on \textit{dpeA} mapping, the strain GJ1075 (carrying \textit{zfl-2102::Tn10dKan} that is closely linked to \textit{dpeB}), was mated with each of the seven Tet\textsuperscript{r} Hfr strains from the mapping kit of Singer et al (207). Exconjugants were selected in the cross by selecting for Tet\textsuperscript{r} colonies and Strep\textsuperscript{r} was used as a contraselection-marker against the Hfr donor. The Tet\textsuperscript{r} Strep\textsuperscript{r} exconjugants were then scored for the loss of the Kan\textsuperscript{r} insertion marker linked to the \textit{dpeB} locus.

The proportion of Kan\textsuperscript{s} amongst the exconjugants obtained from the crosses of GJ1075 with each of the Hfrs CAG5054, CAG5055 and CAG8209 was 1\% (2/175), 19\% (34/178) and 11\% (19/174) respectively. With each of the other four Hfrs, namely CAG5051, CAG5052, CAG5053 and CAG8160 none of 150 exconjugants tested was Kan\textsuperscript{s}. When these results were analysed in the light of the point of
origin and site of Tet\(^r\) insertion in each of the Hfrs, the data could best be explained on the assumption that the Tn\(^{10}\)dKan insertion in GJ1075 is situated between 45 min and 62 min on the E. coli chromosome. To place the site of Tn\(^{10}\)dKan insertion on the chromosome with more precision, the following experiments were done.

### 4.4.1.2 Time-of-entry experiment:

The Tn\(^{10}\)dKan insertion in GJ1075 was transferred by transduction to the Hfr CAG5055 (which, on the basis of the data above, was expected to transfer the dpeB region as a proximal marker), resulting in the strain CAG5055-9. In an interrupted-mating experiment between CAG5055-9 as donor and the multi-auxotrophic strain CSH57 as recipient, aliquots of the mating mixture were taken out at various time-points, vortexed to disrupt the mating pairs and plated for Kan\(^r\), Tet\(^r\) or His\(^+\) exconjugants; Strep\(^r\) was used as the contraselection-marker. CAG5055-9 initiates counterclockwise transfer from its point of origin at 63 min and Tet\(^r\) and His\(^+\) markers are situated at 43 min and 44 min respectively on the linkage map. The numbers of each of the three classes of exconjugants obtained were then plotted as a function of time (Fig. 4.2). It is evident from the plot that the time taken for the entry of the Kan\(^r\) marker into the recipient is approximately 5 minutes. Thus, the Kan\(^r\) insertion marker was placed at around 55- to 59-min on the linkage map.

### 4.4.2 Transduction experiments:

A more accurate map position of zfi-2102::Tn\(^{10}\)dKan was obtained in P1 transduction experiments. Phage P1 lysates grown on each of five strains [from the mapping-collection of Singer et al (207)] carrying Tn\(^{10}\) insertions in this region was used to transduce GJ1075/HYD275 to Tet\(^r\). The transductants from each set were then screened for the Kan\(^8\) phenotype, in order to determine the linkage of zfi-2102::Tn\(^{10}\)dKan with the corresponding Tn\(^{10}\) insertion. The linkage to zfi-2102::Tn\(^{10}\)dKan of zfh-3131::Tn\(^{10}\), srl::Tn\(^{10}\), mutS::Tn\(^{10}\), cysC::Tn\(^{10}\), fuc::Tn\(^{10}\) and recD::Tn\(^{10}\) was found to be 2%, 5%, 49%, 65%, 9% and 4% respectively (Table 4.2).

In order to obtain the linkage values of the dpeB\(^+\) allele itself with each of the above two Tn\(^{10}\)
Fig. 4.2. Conjugational mapping of the zfy-2102::Tn10dKan insertion linked to dpeB. Conjugation between CAG5055-9 as donor and CSH57 as recipient was interrupted at various time points and aliquots of the conjugation mixture was plated on various selective plates. The number of exconjugants obtained are plotted as a function of duration of mating. Symbols: □, Kan Strep; O, Tet Strep; and Δ, His Strep.
**TABLE 4.2. Linkage of different Tn10 insertions to zfj-2102::Tn10dKan and dpeB+ a.**

<table>
<thead>
<tr>
<th>Tn10 insertion (location in min)</th>
<th>b(% Cotransduction frequency between Tn10 and zfj-2102::Tn10dKan)</th>
<th>c(% Cotransduction frequency between Tn10 and dpeB+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>zfj-3131::Tn10 (57.50)</td>
<td>2 (2/93)</td>
<td>10 (9/91)</td>
</tr>
<tr>
<td>srf::Tn10 (58.30)</td>
<td>5 (5/105)</td>
<td>ND</td>
</tr>
<tr>
<td>mutS::Tn10 (58.75)</td>
<td>49 (54/111)</td>
<td>59 (175/297)</td>
</tr>
<tr>
<td>cysC95::Tn10 (59.25)</td>
<td>65 (65/100)</td>
<td>43 (130/310)</td>
</tr>
<tr>
<td>fuc-3072::Tn10 (60.25)</td>
<td>9 (8/91)</td>
<td>5 (5/97)</td>
</tr>
<tr>
<td>recD1901::Tn10 (60.75)</td>
<td>4 (4/97)</td>
<td>2 (2/105)</td>
</tr>
</tbody>
</table>

*a* Donors carried the Tn10 insertions linked to dpeB+. The genotype of the recipient (GJ1075/pHY0275) was proU-lac dpeB zfj-2102::Tn10dKan.

*b* Number of Tet\(^f\) Kan\(^S\)/Number of Tet\(^f\).

*c* Number of Tet\(^f\) dpeB\(^+\)/Number of Tet\(^f\).

ND-Not done.
insertions, the Tet\(^r\) transductants, from the crosses above were also screened for Lac\(^+\) on MacConkey plates supplemented with uracil, Tp, Tet and NaCl (0.15 M). As presented in Table 4.2, \(dpeB\) was linked 59% and 42% to the \(mutS::Tn10\) (58.7 min) and \(cysC::Tn10\) (59.3 min) insertions respectively. Taken together, these results placed \(dpeB\) close to 60 min on the \(E. coli\) chromosome.

4.4.3 Three-factor cross analysis:

**Cross of CAG12173 (cysC::Tn10Kan) into GJ1080/pHYD275 (mutS::Tn10 dpeB):** To determine the relative gene order between \(cysC\), \(dpeB\) and \(mutS\) a cross was set up using CAG12173 (cysC::Tn10Kan \(dpeB\)^\(r\)) as donor and GJ1080/pHYD275 (dpeB mutS::Tn10) as recipient, with selection for Kan\(^r\) transductants. Altogether 310 Kan\(^r\) transductants were scored for their Lac and Tet\(^r\) phenotypes, to determine co-transduction frequencies (in two-factor analyses) between the cysC::Tn10Kan marker on one hand, and \(dpeB\) or \(mutS::Tn10\) on the other. The co-transduction frequencies obtained were 42% and 33% for cysC::Tn10Kan-dpeB and cysC::Tn10Kan-mutS::Tn10 respectively (Table 4.3). The results were also analyzed in the form of a three-factor cross with respect to the markers cys::Tn10Kan, \(dpeB\) and \(mutS::Tn10\), which conclusively established the order of cysC::Tn10Kan-dpeB-mutS::Tn10, at 59 min on the chromosome (Table 4.3).

4.5 \(dpeB\) IS ALLELIC TO \(rpoS\):

An examination of the recent \(E. coli\) linkage map revealed that the gene \(rpoS\) for stationary-phase-specific sigma factor (\(c^S\) or RpoS) of RNA polymerase, lies in the interval between \(cysC\) and \(mutS\) at 59 min. The \(rpoS\) gene was independently discovered in several different contexts by different workers, and was variously designated as \(nur\), \(katF\), or \(appR\). The predicted amino acid sequence of the gene bears strong homology to the family of RNA polymerase sigma factors. Recently, RpoS has been shown to act as a functional sigma factor of core RNA polymerase in an *in vitro* transcription system. Several genes are known to depend on RpoS for their expression, some of which, such as \(otsBA\) (75), \(osmB\) (94) and \(glsG\) (93) are osmotically inducible. The possibility of \(dpeB\) being allelic to \(rpoS\) was thus considered, and the following lines of evidence indicate that this is indeed the case.
TABLE 4.3. Three factor cross analysis of P1(CAG12182) into GJ1080/pHYD275.

Donor genotype (CAG12182): MG1655 dpeB\textsuperscript{+} cysC-3152::Tn\textsubscript{10}Kan

Recepient genotype (GJ1080/pHYD275): GJ134 proU-lac dpeB mutS::Tn\textsubscript{10}.

<table>
<thead>
<tr>
<th>Marker selected (No. of transductants tested)</th>
<th>Class of recombinants</th>
<th>No. of recombinants</th>
<th>Percentage cotransduction freq. between</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) dpeB\textsuperscript{+} Tet\textsuperscript{S}</td>
<td>103</td>
<td>cysC-3152::Tn\textsubscript{10}Kan</td>
<td></td>
</tr>
<tr>
<td>(ii) dpeB\textsuperscript{+} Tet\textsuperscript{r}</td>
<td>26</td>
<td>to dpeB\textsuperscript{+} = 42%</td>
<td></td>
</tr>
<tr>
<td>Kan (310)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii) dpeB Tet\textsuperscript{S}</td>
<td>1</td>
<td>cysC-3152::Tn\textsubscript{10}Kan</td>
<td></td>
</tr>
<tr>
<td>(iv) dpeB Tet\textsuperscript{r}</td>
<td>180</td>
<td>to mutS::Tn\textsubscript{10} = 33%</td>
<td></td>
</tr>
</tbody>
</table>

The least frequent class of recombinant [class (iii)] can be explained as being the product of a four cross over event depicted by symbols 1, 2, 3 and 4 in the fig below. The order of markers thus deduced to be cysC-3152::Tn\textsubscript{10}Kan - dpeB - mutS::Tn\textsubscript{10}. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{diagram.png}
\end{figure}
(i) *E. coli* expresses two catalase isoenzymes, HPI and HPII. *katG* and *katE* are the structural genes for catalase HPI and HPII and are mapped at 89 min and 38 min on the *E. coli* chromosome respectively. HPII synthesis is known to be under RpoS control, and thus *rpoS* strains are phenotypically HPII-negative (138). HPII-positive and -negative strains are easily distinguishable from one another in a qualitative plate assay in which a drop of H$_2$O$_2$ (30% v/v) is placed directly on a colony and the rate of O$_2$ bubble formation on the surface of the colony is monitored. HPII-positive colonies bubble vigorously, whereas HPII-negative colonies exhibit less bubbling (166). In this assay, the *dpeB* derivative of GJ134, GJ1075, behaved like a HPII-negative strain whereas GJ134 itself behaved like a HPII-positive strain.

Furthermore, when a P1 lysate prepared on a *dpeB* zfr-2102::Tn10 dKan donor strain was used to transduce GJ134/pHYD275 to Kan$^\text{T}$, a perfect correlation was demonstrated between segregation of the catalase-negative phenotype (as assayed by the qualitative plate method) and that of the Lac$^-$ phenotype amongst the transductants. Thus, the same *dpeB* mutation was responsible for both the Lac$^-$ and the apparent HPII-negative phenotype.

(ii) That the *dpeB* derivative of GJ134, GJ1075, specifically lacks catalase HPII activity was demonstrated in an experiment in which cell lysates prepared from GJ1075 and GJ134 were electrophoresed under nondenaturing conditions and the gel was stained for catalase activity as described in Chapter 2. The wild-type strain GJ134 was shown to have both catalase HPII and HPI activities, whereas GJ1075 (*dpeB*) specifically lacked catalase HPII activity (Fig. 4.3).

(iii) A known *rpoS* null allele (*rpoS359::Tn10*) from RH90, was introduced by P1 transduction into GJ134/pHYD275. The resultant transductant, GJ1099/pHYD275, was catalase-negative (as seen by the qualitative-plate-assay method) and exhibited similar reduction in proU-lac expression from plasmid pHYD275 as did the *dpeB* mutant GJ1075/pHYD275 (Fig. 4.4).

Taken together, the results of the above experiments indicated that *dpeB* is allelic to *rpoS*. pa
Fig. 4.3. Catalase HPI and HPII activities of various strains. Cell extracts were electrophoresed on polyacrylamide gel under non-denaturing conditions and catalase bands were visualized following activity staining. The bands corresponding to HPI (doublet) and HPII are marked. Lanes: 1, GJ134 (wild-type); 2, GJ1075 (dpeB); and 3, AB1157.
Fig. 4.4. Effect of *rpoS* null mutation on expression of *proU-lac* from plasmid pHYD275. Cultures for assay were grown to mid-log phase in K medium supplemented with various concentrations of NaCl as indicated. Symbols: □, GJ134 (wild-type); O, GJ1075 (*dpeB*); and △, GJ1099 (*rpoS::Tn10*).
In the course of the present studies, the commonly used strain AB1157 was shown to harbor a presumptive rpoS mutation that affects both proU P1 expression (as judged by Lac phenotype on McConkey plates) and enzyme HPII activity (Fig. 4.3) and this mutation was not suppressed by supE.

4.6 GROWTH-PHASE-DEPENDENT EXPRESSION FROM proU P1 PROMOTER:

Several of the RpoS-dependent genes are osmotically inducible and are also growth-phase regulated. These include glgS, otsA, otsB and osmY, all of which are induced in the stationary phase of growth in an RpoS-dependent manner. Since the proU P1 promoter is RpoS-dependent and also osmotically inducible, the effect of growth phase on its expression was examined.

The strain GJ134/pHYD275 (wild-type) was grown in LB medium, and β-galactosidase specific activity (as a measure of lac expression from proU P1 promoter) was measured at various phases of growth. Expression from the promoter P1 was low in log-phase cells (7 U), increased as the cultures entered stationary-phase and reached a level in late stationary-phase (730 U) that was 100-fold higher than the log-phase value (Fig. 4.5). Growth-phase-dependent induction of promoter P1 expression was not observed in a similar experiment with the corresponding dpeB mutant strain GJ1075/pHYD275 (Fig. 4.5).

These results indicate that the P1 promoter is a stationary-phase-specific promoter and that its growth-phase-dependent induction requires RpoS.

In the experiment above, a nutrient-rich medium (LB) was particularly chosen to monitor stationary-phase induction of P1 because it is known from work with other stationary-phase-induced genes that in minimal salts medium the magnitude of such induction is considerably decreased (93, 129). Growth-phase-dependent induction of promoter P1 was also monitored in low-osmolarity K medium which contains Casamino acids, but which is not as nutrient rich as LB. In K medium, promoter P1 expression was higher in the stationary phase of growth than it was in the log-phase of growth, but the level of stationary-
Fig. 4.5. Growth-phase-dependent induction of promoter P1 expression in LB medium. Cultures of GJ134/pHYD275 (wild-type; $\triangle$, $\blacksquare$) and GJ1075/pHYD275 ($dpeB$; $O$, $\bullet$) were grown in LB. Optical densities at 600 nm (open symbols) and $\beta$-galactosidase specific activities (closed symbols) were monitored for each culture at the various stages of growth and the values have been plotted as a function of time.
phase induction seen in this medium (about 4- to 6-fold) was less than that in LB (about 100-fold), and
the induction was also more gradual (Fig. 4.6).

4.7 EXPRESSION OF proU P2 AND P1+P2+NRE AS A FUNCTION OF GROWTH-PHASE:

With the observation that isolated promoter P1 expression is induced in stationary phase even in
low-osmolarity media, it was expected that expression from the full-length proU regulatory region (ie.,
P1R+P2R+NRE) would also be induced under these conditions. To test this possibility, lac expression
from plasmid pHYD272 (P1+P2+NRE) was studied in GJ134 (wild-type) in LB medium at various phases
of growth. Unexpectedly, proU expression exhibited only a marginal increase in the stationary phase
(Fig. 4.7).

One possibility for the lack of stationary-phase-induction of wild-type proU in pHYD272
(P1R+P2R+NRE), in spite of increased expression from isolated promoter P1 (as seen from plasmid
pHYD275) in stationary phase, was that growth phase might have opposing effects on the two promoters
P1 and P2, such that an increase in transcription from promoter P1 in stationary phase is cancelled by a
simultaneous decrease in transcription from promoter P2. To test this possibility, cultures of GJ134 (wild-
type) carrying each of the three plasmids, pHYD275 (P1R), pHYD283 (P2R) and pHYD272
(P1R+P2R+NRE), were grown in K media supplemented with different concentrations of NaCl. Specific
activities of β-galactosidase were measured for the various cultures at different phases of growth, and the
results are presented in Fig. 4.8.

The results may be summerarized as follow: (i) expression from plasmid pHYD272
(P1R+P2R+NRE) was constant over growth phase at all osmolarities tested; (ii) expression from plasmid
pHYD275 (P1R alone) increased with growth phase and this trend was identical for all osmolarities of the
growth media; and (iii) expression from plasmid pHYD283 (P2R alone) was reduced approximately two-
fold as cells approached stationary phase, and this reduction was also seen at all osmolarities of the
Fig. 4.6. Growth-phase-dependent induction of promoter P1 expression in K medium. Culture of GJ134 (wild-type) was grown in K medium. Optical densities at 600 nm (O) and β-galactosidase specific activities (Δ) of the culture were monitored at the various stages of growth and the values have been plotted as a function of time.
Fig. 4.7. Expression of proU-lac from plasmid pHYD272 as a function of growth phase. Culture of GJ134/pHYD272 (wild-type) was grown in LB medium. Optical densities at 600 nm (O) and β-galactosidase specific activities (●) were monitored at various stages of growth and the values have been plotted as a function of time.
Fig. 4.8. Effect of growth phase on proU-lac expression at various osmolarities of the growth medium. β-galactosidase specific activities in cultures of GJ134 (wild-type) carrying pHYD275 (Panel A), pHYD284 (Panel B) and pHYD272 (Panel C) are plotted as a function of optical density at 600 nm of the cultures. Cultures for assay were grown in K medium without (●), with 0.1 M (○), with 0.2 M (□), or with 0.3 M (△) NaCl.
growth media. The possible significance of these results in explaining the absence of \( dpeB \) effect on \( proU \) (P1R+P2R+NRE) regulation is further discussed below.

### 4.8 EFFECT OF MUTATION IN \( hns \) ON P1 PROMOTER EXPRESSION:

Earlier studies in this laboratory had shown that \( lac \) expression from pHYD275 (P1R alone) is reduced in \( hns \) mutants, and the data described in this Chapter indicated that P1 is also under RpoS control. These results demonstrated the existence of several parallels between regulation of the \( proU \) P1 promoter and that of the \( E. \) coli \( csgA \) gene.

The \( csgA \) gene encodes the subunit of curli, a fibronectin-binding protein on the surface of pathogenic \( E. \) coli strains. Expression of \( csgA \) is controlled at the level of transcription by growth temperature and by osmolarity. Olsen et al (172) have also shown that the expression of \( csgA \) is induced in stationary-phase of growth, and that such expression is reduced in \( rpoS \) mutants. \( csgA \) expression is also reduced in a \( hns \) mutant as is expression from the \( proU \) P1 promoter. Interestingly, in a \( hns \) \( rpoS \) double mutant, the level of \( csgA \) expression is higher than that in either of the single mutants, and approaches that in the wild-type strain (172).

To examine whether this combined effect on \( csgA \) of mutations in \( hns \) and \( rpoS \) is extendable to the \( proU \) promoter P1 as well, the following experiment was done. GJ1088/pHYD275 (\( rpoS \) \( hns \)) was constructed by transduction, with a P1 lysate grown on GJ1075 (GJ134 \( dpeB \) \( zfj-2102::Tn10dKan \)) to transduce GJ1083/pHYD275 (GJ134 \( hns \)) to Kan\(^{\text{r}}\). One such Kan\(^{\text{r}}\) transductant that was proved to carry the \( rpoS \) mutation as judged by the qualitative-plate assay for catalase, was designated GJ1088/pHYD275 (\( hns \) \( rpoS \)). Expression from promoter P1, present on the plasmid pHYD275, was monitored in this strain as a function of the osmolarity of the growth medium, and the results are presented in Fig. 4.9. Expression of \( lac \) from pHYD275 in GJ1088 (\( rpoS \) \( hns \)) was identical to that in GJ1099 (\( rpoS \)) and much less than that in GJ1083 (\( hns \)). These data demonstrate that the absence of RpoS results in complete loss of expression from promoter P1 and that, unlike in \( csgA \), introduction of an \( hns \) mutation in this background does not make the promoter \( rpoS \)-independent.
Fig. 4.9. Effect of mutation in hns gene on expression of promoter P1 from plasmid pHYD275. Cultures for assay were grown in K medium supplemented with various concentrations of NaCl as indicated. Symbols: ●, GJ134 (wild-type); □, GJ1075 (dpeB); △, GJ1083 (hns); and ○, GJ1088 (hns dpeB).
4.9 CONSTRUCTION OF A DEFINED dpeA dpeB DOUBLE MUTANT:

The double mutant GJ134 dpeA dpeB (designated GJ1098) was constructed by sequential Plkc transductions with the aid of linked transposon-markers. The profiles of proU-lac expression from the various Tp\textsuperscript{+} plasmids introduced into GJ1098 were indistinguishable from the corresponding derivatives of the originally isolated mutant strain, GJ1055 (Fig. 4.10). GJ1098 also exhibited the DHP\textsuperscript{+} phenotype on NaCl-supplemented minimal A plates to the same extent as GJ1055. These results indicated that dpeA and dpeB are the only two mutations present in GJ1055 that affect proU regulation.
Fig. 4.10. A dpeA dpeB double mutant is similar to the original mutant isolate GJ1055 in proU-lac expression. Derivatives of GJ134 (wild-type, O) GJ1055 (□) and GJ1098 (dpeA dpeB, Δ) carrying the various Tp plasmids (as mentioned for each panel) were grown to mid-log phase in K medium supplemented with various concentrations of NaCl as indicated. Panels: A, pHYD275 (P1R); B, pHYD284 (P2R), C, pHYD283 (P1R+P2R); and D, pHYD272 (P1R+P2R+NRE).
DISCUSSION

As described in chapter 3, the mutant GJ1055 isolated in this study exhibited reduced expression of lac from the P1 promoter of proU. In the same chapter the presence of dpeA (hupB) allele in this mutant strain was demonstrated, but the reduction in P1 expression could not be accounted for solely by the presence of the dpeA allele; the possibility that GJ1055 carries a second mutation affecting promoter P1 expression was thus considered. In this chapter, the existence of such a mutation, designated dpeB, was demonstrated. It was also established that dpeA and dpeB are the only two mutations present in GJ1055 that affect proU regulation. In this section, (i) the effect of dpeB on the various proU cis-regulatory mechanisms; (ii) evidence in support of dpeB being allelic to rpoS, and (iii) the role of RpoS in stationary phase- and osmotic-induction of promoter P1 as well as in regulation of the native proU operon has been discussed.

4.10 dpeB AFFECTS EXPRESSION FROM PROMOTER P1:

The construction of a dpeB single-mutant derivative of GJ134 (GJ1075) was used to show that this mutation alone was sufficient to drastically curtail promoter P1 expression. Comparison of lac expression from the set of four proU-lac plasmids, namely pHYD272, pHYD275, pHYD283 and pHYD284, in the dpeB strain, with that from similar transformants of the wild-type strain suggested that the dpeB mutation specifically affects expression from promoter P1 and that neither P2R nor NRE function was significantly affected by it. Surprisingly, however the dpeB mutation did not affect the overall expression of proU as seen from the plasmid pHYD272 that carries all the three cis-regulatory mechanisms (P1R, P2R and NRE). The lack of dpeB effect on constructs that carry the total proU regulatory region has been discussed later in this section.

4.11 dpeB IS ALLELIC TO rpoS:

Based on the following criteria, the dpeB mutation was shown to be allelic to rpoS, the gene coding for the stationary-phase-specific sigma factor RpoS.
(i) Map position: The dpeB locus was mapped to 59 min by conjugation and Plkc transduction. Two and three-factor analyses of transductional cross data established a gene order of mutS-dpeB-cysC and suggested that dpeB is in or very close to rpoS, the gene coding for the stationary phase specific sigma factor RpoS.

(ii) The dpeB mutant derivative specifically lacked catalase HPII activity, a phenotype characteristic of rpoS strains.

(iii) A known rpoS null allele (rpoS359::Tn10) had the same effect on expression from the proU P1 promoter as did the dpeB mutation.

4.12 GROWTH-PHASE-DEPENDENT EXPRESSION FROM proU P1 PROMOTER:

RpoS-dependent genes are stationary-phase-induced and a subset of RpoS-dependent genes are also osmotically inducible, the most characteristic example of this class being the trehalose-biosynthetic otsBA operon. Expression from the P1 promoter was also found to be markedly induced during entry into stationary phase of growth. Growth-phase-dependent induction of promoter P1 expression was also found to be dependent on the presence of functional RpoS. The rpoS-mediated 100-fold-induction of this promoter is the highest so far known for any stationary-phase-induced gene. This finding also places proU in the class of genes previously shown to be activated both at elevated osmolarity and in the stationary phase.

4.13 RpoS DOES NOT AFFECT OSMOTIC REGULATATION OF P1 EXPRESSION:

As discussed above, although expression from promoter P1 was drastically affected in an rpoS strain, the rpoS mutation did not alter the osmotic induction ratio of expression from this promoter.
Several genes, expression of which are dependent on RpoS, exhibit osmotic inducibility and are also induced in stationary phase of growth in an RpoS-dependent fashion. Hengge-Aronis et al (94, 95) had employed instantaneous induction assays to show that several genes including otsBA exhibit loss of osmotic inducibility in an rpoS background. On the other hand, Kaasen et al (119) showed that osmotic inducibility of otsBA during steady-state growth was not completely abolished in rpoS mutants even though basal expression was considerably reduced; the findings of this study on steady-state proU P1 expression are similar to those of Kaasen et al (119).

These observations above, that there is an overlap between the gene-induction responses to osmotic stress and to starvation, would seem to implicate rpoS as an osmoregulatory gene. However, the following arguments suggest that RpoS is unlikely to be directly involved in osmoregulation. (i) If one assumes that RpoS is involved in both stationary-phase- and osmotic-induction of gene expression, it is difficult to explain how, for a given gene, the levels of induction by osmotic stress and by growth phase do not correlate. For example, g1gS and proU P1 exhibit, respectively, about 50- and 100-fold induction in stationary phase, whereas they are induced only about 2- and 4-fold by osmotic stress. (ii) Not all ApeS-dependent genes are osmotically inducible. On a two-dimensional gel analysis of total cellular proteins, expression of several RpoS-dependent proteins was found not to be affected by increased osmolarity (95). (iii) In case of the heat-shock regulon, the expression of heat-shock genes seems to be directly proportional to the cellular concentration of the heat-shock sigma-factor σ^{32} (88). Likewise, there is an increase by several-fold of the expression of rpoS at both the level of transcription and translation in stationary-phase cultures (129, 139, 167), but osmotic stress has only a minor effect (two-fold) on RpoS synthesis (95).

Thus it seems likely that within the rpoS regulon there exists a subset of genes whose expression is induced by osmotic stress. Although the corresponding promoters require RpoS directly or indirectly for transcription, their osmotic regulation appears to be achieved not by a change in the total cellular concentration of RpoS but by some other factors.
4.14 A CONSENSUS SEQUENCE FOR RpoS-CONTROLLED PROMOTERS:

No consensus sequence motif for RpoS-controlled promoters has so far been identified, although promoter sequences of several RpoS-dependent genes and their transcriptional start sites are known including those for *katE*, *xthA*, *bolA*, *appY*, *treA*, *csgA*, *mcc* and *glgS*. Based on in vitro studies using purified RpoS and core RNA polymerase, Tanaka et al. (219) have suggested the following classification of promoters in *E. coli*: (i) type I, that are recognized by both $\sigma^{70}$ and $\sigma^{8}$ factors of RNA polymerase; (ii) type II, that are recognized only by $\sigma^{70}$; and (iii) type III, that are recognized only by $\sigma^{8}$. Existence of promoters that are recognized by both $\sigma^{70}$ and $\sigma^{8}$ suggested that a consensus promoter sequence for $\sigma^{8}$ might not be too different from the $\sigma^{70}$ consensus. An examination of promoter sequence of these three classes revealed that type I and type II promoters are similar to one another in their sequences in the -35 and -10 regions whereas type III promoters appear to have a very different -35 region.

The *katE* gene, whose expression in vivo depends on RpoS, could not be transcribed in vitro by RNA polymerase holoenzyme with $\sigma^{8}$ (219), suggesting that the in vivo effect of *rpoS* mutation on *katE* may be indirect. Consistent with the notion that the in vivo effect of *rpoS* mutation on several genes might involve a sequential cascade of regulatory factors, is the finding that the products of the RpoS-controlled genes, *bolA* and *appY* themselves have regulatory functions.

A comparison undertaken as part of the present study, of the sequence in the vicinity of *proU P1* promoter with that of the *glgS P2* promoter (which is also RpoS-controlled) revealed a 5-base- and a 9-base-stretch of identical nucleotides, situated in the -35 and -10 regions respectively (Fig. 4.11). It is possible that these sequences, or some parts thereof, might represent a consensus motif for *rpoS*-controlled promoters.

4.15 IS THERE AN OVERLAP BETWEEN RpoS AND HNS MEDIATED GENE REGULATION?

The *csgA* gene encodes a subunit of the curli protein, which is involved in binding of cells to
Fig. 4.11. Allignment of the *E.coli* (*E. c*) proU P1 and glgS P2 promoter sequences, upstream of transcription initiation site (+1). The bases of the -10 and -35 regions that are identical are shown in upper case, as are the other positions of sequence matches between the promoters. Also shown is the corresponding region (designated P1) of the *S. typhimurium* (*S. t*) proU sequence, for which the bases that are identical with the pair above are marked in upper case; the +1 start site of transcription marked in this instance is putative.
fibronectin in mammalian tissues. Expression of csgA is dependent on RpoS, is repressed by mutation in hns and is growth-phase-regulated (172). Interestingly, mutations in hns result in derepression of csgA expression in rpoS-deficient strains. These results led Olsen et al (172) to suggest that in case of csgA, the role of RpoS is to relieve HNS-mediated repression.

In many respects, proU P1 expression is affected in a manner similar to csgA by mutation in rpoS or hns and by growth phase. However, P1 promoter expression was not derepressed by an hns mutation in an rpoS background, suggesting that the model proposed above may not be generally applicable.

4.16 RpoS AND NATIVE proU EXPRESSION:

Although the effect of the dpeB (rpoS) mutation on lac expression from plasmid pHYD275 (carrying P1R) was quite pronounced, that on expression from plasmid pHYD272 (carrying P1R+P2R+NRE) was not significant. Kassen et al (119) have also shown earlier that an rpoS mutation does not significantly affect expression of a normal chromosomal proU-lac fusion. Furthermore, the studies described in this chapter have demonstrated that unlike the isolated promoter P1, which is strongly induced during entry into stationary phase, constructs carrying P1R+P2R+NRE exhibit no significant changes in expression as a function of growth phase. This lack of dpeB and growth phase effects on native proU expression could be due to either of two reasons: (i) Promoter P1 of proU is not relevant in the osmotic regulation of proU expression; or (ii) P1 does play a role in osmotic induction of proU expression, but the effects of growth phase or dpeB mutation on native proU is a result of essentially opposing effects of these two factors on the P1 and P2 promoters. Each of these possibilities is discussed below.

4.16.1. Is promoter P1 relevant in osmotic regulation of proU?

This question becomes important because of two sets of observations:

(i) Although the existence of P1 promoter of proU in E. coli was reported earlier by workers from this laboratory, others have not detected the presence of such a promoter for the otherwise similar proU operon of S. typhimurium. pa (ii) Several workers have argued that the expression of proU is solely
dependent on the vegetative sigma factor of RNA polymerase, that is $\sigma^{70}$. In vitro transcription studies by Prince and Villarejo (185) using purified RNA polymerase have been cited to imply that proU expression depends on $\sigma^{70}$-containing holoenzyme. Yim et al (242) have also suggested that osmotic inducibility of proU is solely dependent on the $\sigma^{70}$ factor of RNA polymerase, as proU expression upon osmotic upshock was considerably reduced at restrictive temperature in a rpoD (Ts) strain that makes a temperature-sensitive $\sigma^{70}$-polypeptide.

However, these two sets of observations are inconclusive, in deciding upon the relevance of the P1 promoter, for the following reasons.

(a) The inability of other workers to identify an equivalent promoter upstream of proU in S. typhimurium might perhaps be explained on the assumption that they had inadvertently been working with rpoS mutant derivatives, as has been shown to be the case with many laboratory strains of E. coli (119). In this context, it is also worth mentioning that: (i) in the course of the present studies, the commonly used strain AB1157 was found to harbour a presumptive (and previously uncharacterised) rpoS mutation at 59 min that is not suppressed by supE44 and that affects both proU P1 expression and enzyme HPII activity; and (ii) there is significant conservation of sequence in the appropriate region of S. typhimurium proU (ie., the putative promoter region) with the pair of shared stretches identified above between E. coli proU P1 and glgS P2 (Fig. 4.11).

(b) In the in vitro transcription studies on proU the possibility of existence of two promoters, one (P2) dependent on $\sigma^{70}$ and other (P1) on $\sigma^8$, was not taken into account in the interpretation of the data.

(c) In the studies of Yim et al (242), proU expression was assayed in a rpoD (Ts) strain that was subjected simultaneously to osmotic-upshock and to heat shock (to 44°C), and it is not clear to what extent proU inducibility might have been affected by the latter treatment. Furthermore, even under these conditions, there did exist a residual expression and 4-fold osmotic induction of proU which could possibly represent the contribution of the RpoS-controlled P1 promoter and the associated P1R mechanism.
Additionally, the following lines of evidence indicate that the P1 promoter is indeed probably relevant in \textit{proU} osmotic regulation: (i) The \textit{dpeB} mutation (which affects only promoter P1) was identified in a selection for derivatives with reduced \textit{proU}\textsuperscript{+} expression, and presumably therefore must have contributed to the selective growth advantage of the mutant strain. (ii) The present data on P1 regulation are also in consonance with the overlap that has been demonstrated by other workers between osmotic and stationary-phase response for the promoters of several other loci in \textit{E. coli}.

4.16.2. Do growth phase and \textit{rpoS} have opposing effects on the two promoters of \textit{proU}?

In the light of growth-phase-regulation of promoter P1 and of the pronounced effect of mutations in \textit{rpoS} on promoter P1 expression, two things remain unexplained: (i) the lack of stationary-phase-induction of the native \textit{proU}; and (ii) the lack of effect of mutation in \textit{rpoS} on \textit{proU} expression in its native context.

In cultures grown in K medium containing various concentrations of NaCl, expression from P1 (in plasmid pHYD275) increased and that from P2 (in plasmid pHYD284) decreased steadily with increasing optical density of the cultures. At certain osmolarities the effect of growth phase on the two isolated promoters was opposite and equal in magnitude, so that the sum of \(\beta\)-galactosidase specific activities for cultures of the two plasmid carrying strains remained constant over growth phase. Furthermore, expression from plasmid pHYD272 (possessing P1R+P2R+NRE) decreased only marginally over growth phase at all osmolarities. One possibility could therefore be that growth phase has opposing effects on the two \textit{proU} promoters, and that non-induction of native \textit{proU} in stationary phase may be a result of the canceling effect between increase in P1 expression and decrease in P2 expression in stationary phase. It must be mentioned here, however, that expression from plasmid pHYD283 (carrying P1R+P2R) does not appear to be a simple sum of that from plasmids carrying the promoters individually. On the other hand, it is also possible that the reason for the lack of any effect of mutations in \textit{rpoS} on native \textit{proU}
expression may be more complex. Thus, mutation in rpoS could lead indirectly to increased expression from promoter P2, through the former’s effect on cellular osmoregulation. Mutation in rpoS does interfere with other osmotically regulated genes that play a role in restoring cell turgor under conditions of osmotic stress, (for example, the otsBA operon involved in trehalose biosynthesis). Thus, for a given level of osmotic stress, an rpoS mutant strain may be expected to experience a greater decrease in turgor pressure compared to its wild-type parent, and consequently to exhibit higher expression of osmoreponsive genes. The lack of rpoS effect on native proU expression can thus perhaps be explained by an increased P2 expression (due to reduced turgor) in the mutant strain, which in effect cancels out the decrease in promoter P1 expression.

4.17 IS proU A VIRULENCE GENE?

The presence of osmoprotective agents such as glycine betaine and proline betaine in urine (27) renders it likely that proU function is important for colonization by and growth of E. coli in the high-osmolality-niche of the human urinary tract (38). Several genes that affect proU regulation, including hns (51, 91, 99, 184, 222), rpoS (65, 170), and himA/D (144) are known to be regulators of other virulence genes in the enterobacteria. It is therefore tempting to speculate that proU may also be a virulence gene required for intracellular growth of the enteropathogens and it would be interesting to determine whether the proU promoter is also active in the mammalian intracellular milieu (144).