Molecular Cloning, Physical Mapping and Expression of the bet Genes Governing the Osmoregulatory Choline–Glycine Betaine Pathway of Escherichia coli

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An analysis of the bet genes governing the osmoregulatory choline–glycine betaine pathway of Escherichia coli was performed. A 9 kb BamHI fragment, located 30 to 39 kb counterclockwise of the EcoRI site of fad, coded for all known Bet activities. The following genes were identified: the betA gene for the choline dehydrogenase, the betB gene for the betaine aldehyde dehydrogenase, and the betT gene or operon for the high-affinity choline transport. The betB and the betT genes were named in this paper, and the clockwise gene order was shown to be betA,B,T. Subcloning gave plasmids which expressed each of the three Bet activities separately. The cloned bet genes remained osmotically regulated, indicating the existence of several osmotically regulated promoters in the bet region. Salmonella typhimurium, which carried the bet region of E. coli in the broad-host-range vector pRK293 expressed the three Bet activities and displayed increased osmotic tolerance in the presence of choline.

INTRODUCTION

Escherichia coli can overcome growth inhibition caused by osmotic stress by uptake or synthesis of glycine betaine (Le Rudulier et al., 1984; Strøm et al., 1986). The relative contribution of glycine betaine to the osmotic strength of the cytoplasm increases with the external osmotic strength, amounting to nearly 60% in minimal medium of 1.47 osmolal (Larsen et al., 1987). The osmolyte glycine betaine has also been found in many halotolerant heterotrophic (Imhoff & Rodriguez-Valera, 1984; Shked-Vinkler & Avi-Dor, 1975) and phototrophic (Galinski & Trüper, 1982; Reed et al., 1986) bacteria, and in marine animals and halophytic plants (Yancey et al., 1982).

For the synthesis of glycine betaine, E. coli depends on an exogenous supply of choline or (glycine) betaine aldehyde. The organism has a high-affinity and a low-affinity transport system for choline with \( K_m \) values of 8 \( \mu \text{M} \) and 1.5 \( \text{mM} \), respectively. These systems are only expressed under osmotic stress (Styrvold et al., 1986). The organism has a membrane-bound, \( O_2^- \)-dependent, electron-transfer-linked choline dehydrogenase which oxidizes choline to betaine aldehyde, and betaine aldehyde to glycine betaine at about the same rate. In addition, the organism has a soluble NAD-dependent betaine aldehyde dehydrogenase. Full expression of these dehydrogenase activities is found in cells which are grown aerobically in a choline-containing minimal medium of elevated osmotic strength (Landfald & Strøm, 1986). Experiments with chloramphenicol indicate that the transport systems and the dehydrogenases are osmotically regulated at the level of gene expression.

The bet genes which govern the choline–glycine betaine pathway, including the high-affinity choline transport system, cluster at about 7 min on the chromosomal map of E. coli, i.e. counterclockwise to lac (Styrvold et al., 1986). The closely related organism Salmonella typhimurium lacks the lac region of the chromosome and cannot produce glycine betaine from...
choline. Thus, unlike E. coli, S. typhimurium is not protected against osmotic stress by an exogenous supply of choline (Stram et al., 1986; present data). Both organisms are, however, protected against osmotic stress by an exogenous supply of glycine betaine which is taken up by the ProU and ProP systems (Cairney et al., 1985a, b; May et al., 1986).

There are few gene markers in the bet-lac region of the E. coli chromosome, and a genetic analysis of the bet system is also hampered by the ability of choline dehydrogenase to oxidize both choline and betaine aldehyde and by the existence of two choline transport systems (Landfald & Strøm, 1986; Styrvold et al., 1986). In this investigation we have characterized the bet genes by molecular cloning and physical mapping. We also report that the bet genes of E. coli can be expressed in S. typhimurium.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used are listed in Table 1. The minimal medium used was medium 63, containing 100 mM-KH₂PO₄, 75 mM-KOH, 15 mM-(NH₄)₂SO₄, 1 mM-MgSO₄, 4 μM-FeSO₄ and 22 mM-glucose (Miller, 1972). The osmotic strength of the growth medium was increased by addition of 0.4 to 0.75 M-NaCl, and choline (1 or 2 mM) was added as an osmoprotectant, as stated below. The pH was adjusted to 7.2 with NaOH when necessary. Bacteria were grown aerobically at 37 °C, and those used in the biochemical tests were harvested in exponential growth and treated as described by Landfald & Strøm (1986).

**Antibiotics.** The following concentrations (μg ml⁻¹) of antibiotics were used in the growth media where

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Construction or source†</th>
</tr>
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<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSH7</td>
<td>F⁻ lacY rpsL thi</td>
<td>Cold Spring Harbor strain (Miller, 1972; J. Kjøsbakken)</td>
</tr>
<tr>
<td>MC4100</td>
<td>F⁻ araD139 ΔargF⁻ lac U169 fiβ5301 ptsF25 relA1 rpsl150 deoCl</td>
<td>CGSC 6152, B. J. Bachmann</td>
</tr>
<tr>
<td>JC10240</td>
<td>HfrPO45 srl-300 : : Tn10 recA56 relA1 thr-300 ilv-318 rpsE2300 spoT1 thi-1</td>
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<td>FF1000</td>
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<td>JC10240 × MC4100</td>
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<td>FF1000 Δ(srl-300 : : Tn10)</td>
<td>Spontaneous Tc³ mutant</td>
</tr>
<tr>
<td>FF481</td>
<td>F⁻ rpsL thi betA2 srl-300 : : Tn10 recA56</td>
<td>Styrvold et al. (1986)</td>
</tr>
<tr>
<td>BHB2688</td>
<td>recA (λ imm434 cts b2 red3 Eam4 Sam7)</td>
<td>Hohn (1979)</td>
</tr>
<tr>
<td>BHB2690</td>
<td>recA (λ imm434 cts b2 red3 Dam15 Sam7)</td>
<td>Hohn (1979)</td>
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<td>S. typhimurium</td>
<td></td>
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</tr>
<tr>
<td>LT2</td>
<td>Wild-type</td>
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<td>L72 Rif⁸</td>
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<tr>
<td><em>Plasmid</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBR322</td>
<td>ApR TcR</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pBR328‡‡‡∥</td>
<td>ApR TcR CmR</td>
<td>Soberon et al. (1980)</td>
</tr>
<tr>
<td>pRK293</td>
<td>TcR KmR</td>
<td>Ditta et al. (1985)</td>
</tr>
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<td>pLG339</td>
<td>TcR KmR</td>
<td>Stoker et al. (1982)</td>
</tr>
<tr>
<td>cos4</td>
<td>Ap⁸</td>
<td>Roberts et al. (1986)</td>
</tr>
<tr>
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</tr>
<tr>
<td>pFF121</td>
<td>Ap⁸ vector, cos4</td>
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</tr>
<tr>
<td>pFF221</td>
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<td>Km⁸; vector, pLG339</td>
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<tr>
<td>pFF600</td>
<td>Km⁸; vector, pRK293</td>
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</table>

† CGSC, E. coli Genetic Stock Center, Yale University, New Haven, Conn., USA.
‡‡‡ This pBR328* carried an alteration (see text).
appropriate: ampicillin (Ap), 100; chloramphenicol (Cm), 30; kanamycin (Km), 60; rifampicin (Rif), 50; and tetracycline (Tc), 10.

Genetic procedures. Transformation was done using a standard CaCl₂ method; isolation of plasmid and chromosomal DNA, and restriction cleavage and ligation of DNA were done essentially as described by Maniatis et al. (1982). The broad-host-range plasmid pFF600 was transformed into E. coli S17.1 which carries the mob genes of plasmid RP4 in the chromosome (Simon et al., 1983), and then transferred by conjugation into S. typhimurium FF1001. Deletion of transposon Tn10 from FF1000 was done using the method described by Bochnert et al. (1980); the resulting strain FF2000 was used when a Tc<sup>+</sup> host was required.

Gene library and cloning of the bet genes. The plasmids used in this work are listed in Table 1. The cosmid cloning was done as described by Roberts et al. (1986). Plasmid cos4 was linearized by cleaving with PstI and the blunt ends dephosphorylated, followed by cleavage with BamHI to give two 'arms' each containing cos sites. Chromosomal DNA from strain CSH7 was treated with Sau3A to generate fragments of 35 to 45 kb and ligated to the arms of cos4. Recombinant molecules were packed into lambda heads using strains BHB2688 and BHB2690 essentially as described by Hohn (1979). Plasmids were introduced into FF1000 using standard infection procedure, and clones were selected on agar plates containing medium 63 plus 0.7 M-NaCl and 1 mM-choline.

Subcloning of the bet genes. Plasmid pFF21, selected in the cloning described above, had two restriction sites for HindIII, one in the cos4 vector and one in the chromosomal insert of 42.5 kb (Fig. 1). pFF121 was prepared by cleaving pFF21 with HindIII followed by recircularization. pFF221 was constructed by inserting a 90 kb BamHI chromosomal fragment from pFF121 into the BamHI site of pBR328 (Fig. 1). As our pBR328<sup>*</sup> vector appeared to carry an alteration in the TcR gene, the vector part of pFF221 had lost the HindIII, ClaI and EcoRV sites in this region, and gained one SalI site which was located near the left end of the vector in the linearized form of pFF221 depicted in Fig. 1. Thus, pFF600 was constructed by cleaving pFF221 with SalI and subcloning the chromosomal fragment into the SalI site of pBR293 (Ditta et al., 1985). For construction of pFF504, the 5.5 kb BglII–BamHI chromosomal fragment of pFF221 was subcloned into the BamHI site of pLG339 (Stoker et al., 1982).

From pFF221 (Fig. 1), the shortened versions pFF321 and pFF323 were prepared by cleaving vector and insert of pFF221 with EcoRI or PstI, respectively, followed by recircularization. Four chromosomal fragments of pFF221, i.e. a 2.7 kb EcoRI fragment, a 3.3 kb BamHI–PstI fragment, a 3.7 kb PstI–EcoRI fragment and a 2.5 kb PstI–PstI fragment, were subcloned into the corresponding restriction sites of pBR322. The resulting plasmids were pFF222, pFF326, pFF423 and pFF424, respectively. (The chromosomal DNA present in all subclones of pFF221 is shown in Fig. 2.)

In vivo choline oxidation test. The oxidation of [¹⁴C]choline to [¹⁴C]glycine betaine by cells growing in medium 63 plus 0.6 M NaCl was measured as described by Styrvold et al. (1986).

Enzyme assays. Choline dehydrogenase activity was measured radiochemically at 37 °C using toluene-treated cells. NAD-dependent betaine aldehyde dehydrogenase activity was assayed spectrophotometrically at 340 nm at room temperature (22 °C) using the 12000 g supernatant of cells disrupted in a miniature French pressure cell (American Instruments). All procedures were as described by Landfald & Strom (1986).

Choline uptake assay. This was done using the radiochemical filtration method as described by Styrvold et al. (1986). Unless stated otherwise, cells were grown and tested in medium 63 plus 0.4 M-NaCl and, in order to inhibit protein synthesis, chloramphenicol (100 µg ml⁻¹) was added 3 min prior to their use.

Radiochemicals and other methods. The CodAB phenotype was determined by growth on medium 63 agar containing 100 µg 5-fluorocytosine ml⁻¹. Cod<sup>+</sup> mutants grow in the presence of this analogue, unlike Cod<sup>+</sup> cells (Ahmad & Pritchard, 1972; Styrvold et al., 1986). Radiochemicals and other methods were described previously (Landfald & Strom, 1986; Styrvold et al., 1986).

RESULTS

Molecular cloning of the bet–codAB region

The deletion mutant MC4100 Δ(argF–lac) lacks the bet genes which govern high-affinity choline uptake, choline dehydrogenase and betaine aldehyde dehydrogenase. MC4100 has, however, retained a rapid low-affinity uptake system for choline which has not been characterized genetically (Styrvold et al., 1986). Strain CSH7, which can produce the osmoprotectant glycine betaine from choline, grows on agar plates with medium 63 containing 0.7 M NaCl and 1 mM-choline (i.e. Bet<sup>+</sup> phenotypic growth on salt agar), whereas MC4100 does not grow under these conditions. The codA and codB genes which code for cytosine deaminase and uptake, respectively, are the only known gene markers in the bet–lac region, and we have previously established that the clockwise gene order is bet–cod–lac (Styrvold et al., 1986). Cod<sup>+</sup> mutants, such as MC4100, are resistant to 5-fluorocytosine (Ahmad & Pritchard, 1972; Styrvold et al., 1986).
We prepared a gene library of CSH7 in the cosmid vector cos4; the chromosomal DNA was partially digested with Sau3A and ligated into the BamHI site of the vector. By direct plating of infected cells of FF1000 (i.e., a recA mutant of MC4100) on the choline-containing salt agar, we selected ApR strains with Bet+ growth characteristics. Plasmid pFF21, isolated from a strain displaying Bet+ Cod+ Lac- growth phenotype, was selected for further characterization. A restriction map of this plasmid, which carried a 42.5 kb chromosomal DNA insert, is shown in Fig. 1. When a 18.3 kb chromosomal fragment of the right side of pFF21 was removed, the Cod+ phenotype was lost. (The resulting plasmid was pFF121, see Methods.) Furthermore, when the left 9.0 kb chromosomal BamHI fragment of pFF21 was subcloned into a pBR328* vector, the resulting plasmid pFF221 (Fig. 1) expressed all three Bet activities.

Comparing the restriction map of pFF21 (Fig. 1) with the restriction map of the proA–purE region published by Hadley et al. (1983), it appeared that the right EcoRI site of pFF21 corresponds to the only EcoRI site of the lacZ gene. Thus, the BamHI fragment carrying the bet region is located 30 to 39 kb counterclockwise of this restriction site.

Subcloning of the bet region

Starting from the plasmid pFF221 we prepared several subclones of the cloned 9.0 kb chromosomal BamHI fragment. The fragments present in the subclones are shown in Fig. 2 (the right side of the restriction map is proximal to the lac operon), and the vectors used are listed in Table 1. The data summarized in Fig. 2 showed that one plasmid was constructed (i.e. pFF321) which coded for both of the dehydrogenase activities, but not the choline uptake activity, and several plasmids were isolated which coded for each of the three Bet activities separately.

Plasmids with the left BamHI–PvuII fragment coded only for the choline dehydrogenase (Fig. 2). To ensure that the gene(s) of this dehydrogenase was transcribed from the native promoter and not from a vector promoter, we constructed two plasmids which gave different flanking regions for this chromosomal fragment. These plasmids, which gave similar results in the biochemical tests, were pFF323 with the vector pBR328 and pFF326 with the vector pBR322. It should be noted that the BamHI region of the two vectors is identical, but the constructs have retained different parts of the TcR gene. Plasmid pFF423, containing the more central bet region, coded only for the betaine aldehyde dehydrogenase whereas plasmid pFF504, containing the left BglII–BamHI region, coded only for the high-affinity choline transport activity. Apparently, the gene(s) governing this transport system, are located near the more central EcoRI site. All subclones lacking the region to the right of this site and plasmid pFF222 carrying the 2.7 kb EcoRI fragment did not code for the transport activity (Fig. 2).

Expression of the Bet dehydrogenase activities

Table 2 lists the Bet dehydrogenase activities of the parental strains CSH7 and MC4100, and of MC4100 recA strains carrying plasmids with various parts of the bet region. Probably as a result of increased gene dosage, the dehydrogenase activities of the plasmid-carrying strains
Osmoregulatory bet genes of Escherichia coli

Fig. 2. Subcloning of the bet region of E. coli. The top line shows a restriction map of the 9.0 kb chromosomal DNA of plasmid pFF221, and below the extent of the chromosomal DNA of various subclones is shown. The symbols for the restriction sites are given in the legend to Fig. 1. The activities expressed by the plasmids in a Δ(argF-lac) background (i.e. FF1000 and FF2000) are shown on the right side. CDH (BetA) is choline dehydrogenase, BDH (BetB) is betaine aldehyde dehydrogenase and Cup (BetT) is high-affinity choline uptake. The approximate location of the genes is indicated above the restriction map. It should be noted that the chromosomal fragment of pFF221 contained an additional EcoRI site which was located in the EcoRI fragment shown in the Figure. Two of the sites were separated by 350 b, but we do not know whether the third site was located on the left or right side. However, this uncertainty did not influence the interpretation of the data. (The left side of the restriction map is in the counterclockwise direction on the chromosomal map.)

Table 2. Choline dehydrogenase and betaine aldehyde dehydrogenase activities of CSH7 and bet deletion mutants with and without high-copy-number plasmids carrying the bet genes

Uninduced cells were grown aerobically in medium 63 and stress-induced cells were grown aerobically in medium 63 plus 0.4 M-NaCl (without choline). The cells were harvested in the exponential growth phase. Choline dehydrogenase (CDH) activity was measured radiochemically at 37 °C using toluene-treated cells. Betaine aldehyde dehydrogenase activity (BDH) was measured spectrophotometrically at 22 °C using crude cell extract. The figures are means of three independent measurements; the SE values were within ±15%.

<table>
<thead>
<tr>
<th>Strain(plasmid)*</th>
<th>CDH</th>
<th>BDH</th>
<th>CDH</th>
<th>BDH</th>
</tr>
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<tbody>
<tr>
<td>CSH7</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>MC4100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FF1000(pFF221)</td>
<td>10</td>
<td>34</td>
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<td>300</td>
</tr>
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<td>FF1000(pFF321)</td>
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<tr>
<td>FF2000(pFF423)</td>
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<td>65</td>
<td>0</td>
<td>240</td>
</tr>
</tbody>
</table>

* The gene library was prepared from strain CSH7. Strains FF1000 and FF2000 are recA mutants of MC4100 Δ(argF-lac).

were always higher than those of CSH7. Furthermore, the dehydrogenase activities were two to ninefold higher when the cells were grown under osmotic stress (medium 63 plus 0.4 M-NaCl) than when they were grown without stress (medium 63 alone). The apparent osmotic induction was in most cases higher for the soluble betaine aldehyde dehydrogenase than for the membrane-bound choline dehydrogenase.
Cells of FF1000(pFF326) and FF2000(pFF423) transferred from medium 63 to medium 63 plus 0.65 M-NaCl and incubated aerobically for 5 h at 37 °C, displayed fully induced activity of choline dehydrogenase or betaine aldehyde dehydrogenase, respectively. When the osmotic upshock was done in the presence of chloramphenicol (150 µg ml⁻¹), there was essentially no increase in the specific dehydrogenase activity of the cells above the level found at low osmotic strength. This indicated that the osmotic induction required *de novo* protein synthesis.

The presence of choline (1 mM) in the growth medium stimulated the synthesis of the dehydrogenases in stressed cells of strain K10 (Landfald & Strøm, 1986). In the present investigation we found that the addition of choline gave a twofold increase in the betaine aldehyde dehydrogenase activity of osmotically stressed cells of FF2000(pFF423). They synthesized only this dehydrogenase and did not produce glycine betaine. However, all osmotically stressed cells which carried high-copy-number plasmids coding for the choline dehydrogenase displayed a 40 to 60% reduction of both dehydrogenase activities when grown in the presence of choline (data not shown).

It is also noteworthy that strains which synthesized only the choline dehydrogenase [i.e. FF1000(pFF323) and FF2000(pFF326)] were protected against osmotic stress by choline and also oxidized [¹⁴C]choline to [¹⁴C]glycine betaine *in vivo*. In this respect they behaved similarly to the strains which synthesized both dehydrogenases (data not shown). This supported the notion that the choline dehydrogenase can oxidize both choline and betaine aldehyde (Landfald & Strøm, 1986; Styrvold *et al.*, 1986). Deletion of the cloned high-affinity transport system did not influence osmoprotection by choline, since the hosts carried a second chromosomally encoded transport system for choline (see above). As would be expected, pFF323 and pFF326 also complemented mutant FF481 which carries a mutation in the *betA* gene which encodes choline dehydrogenase (Styrvold *et al.*, 1986).
Osmoregulatory bet genes of *Escherichia coli*

**Fig. 4.** Genetic engineering of osmotic tolerance in *S. typhimurium*; growth stimulation by choline of osmotically stressed cells of *S. typhimurium* FF1001(pFF600). Plasmid pFF600 consisted of the vector pRK293 with the *bet* genes of *E. coli*. Growth of FF1001(pFF600) (○) and FF1001(pRK293) (■) in medium 63 plus 0.75 M-NaCl and 2 mM-choline and of FF1001(pFF600) (▲) in the same medium without choline is shown.

**Fig. 5.** Osmotic-stress-dependent uptake of choline in *S. typhimurium* FF1001(pFF600) carrying the cloned *bet* genes of *E. coli*. Uptake of choline (5 μM) by FF1001(pFF600) which was grown and assayed in the absence of osmotic stress (▲), and by FF1001(pFF600) (■) and *E. coli* FF1000(pFF600) (●) which were induced by growth in medium 63 plus 0.4 M-NaCl and then given chloramphenicol (100 μg ml⁻¹) and 5 μM-choline, in that order, is shown.

*Expression of high-affinity choline transport activity*

Strains such as FF1000, which lack the high-affinity choline transport system, do not display any choline uptake at a concentration of 5 μM (Styrvold et al., 1986). In the uptake experiments depicted in Fig. 3, we used strain FF1000 carrying the low-copy-number plasmid pFF504. This plasmid did not express any of the Bet dehydrogenase activities (Fig. 2). It should be noted that cells of FF1000(pFF504), which were grown in the presence of 0.4 M-NaCl and then treated with chloramphenicol (100 μg ml⁻¹), displayed an immediate uptake of added choline (5 μM). Cells grown without osmotic stress in medium 63 displayed delayed uptake of choline after addition of 0.4 M-NaCl and 5 μM-choline. When this upshock experiment was done in the presence of chloramphenicol, the extent of choline uptake was strongly reduced. Furthermore, in the absence of osmotic stress there was no choline uptake (Fig. 3). This pattern of osmotic induction of choline uptake was exactly the same as that found previously for CSH7 and K10 (Styrvold et al., 1986).

*Expression of the bet genes in S. typhimurium*

A 9.6 kb *Sal*I fragment which encompassed the chromosomal *Bam*HI fragment of pFF221 (see Fig. 1), and thus contained all of the known *bet* genes, was subcloned into the *Sal*I site of the *Te*α region of the broad-host-range vector pRK293. This plasmid, pFF600, was mated into *S. typhimurium* FF1001. Strain FF1001(pFF600) grew in medium 63 plus 0.75 M-NaCl and 2 mM-choline, whereas the parental strain FF1001 with the vector pRK293 did not grow. FF1001 (pFF600) did not grow under osmotic stress (0.75 M-NaCl) in the absence of choline (Fig. 4).
S. typhimurium FF1001(pFF600) and E. coli FF1000(pFF600), grown in medium 63 plus 0.5 M-NaCl, displayed identical dehydrogenase activities: choline dehydrogenase activity was 10 nmol (mg protein)⁻¹ min⁻¹ (at 37 °C) and betaine aldehyde dehydrogenase activity was 60 nmol (mg protein)⁻¹ min⁻¹ (at 22 °C). S. typhimurium FF1001(pRK293) did not show either activity. Furthermore, osmotically stressed cells of FF1001(pRK293) failed to take up choline at 5 μM, whereas FF1001 (pFF600) displayed high-affinity choline uptake activity similar to that of E. coli FF1000(pFF600) (Fig. 5).

DISCUSSION

The present data show that the bet genes, which encode choline dehydrogenase, betaine aldehyde dehydrogenase and the high-affinity transport system for choline, cluster within a 9.0 kb BamHI fragment located 30 to 39 kb counterclockwise of the EcoRI site of the lacZ gene. From this 9-0 kb BamHI fragment, we have isolated subclones which expressed each of the three Bet activities separately, and we have shown that deletion mutants of E. coli carrying these genes on high-copy-number plasmids displayed higher dehydrogenase activities than the wild-type. Furthermore, S. typhimurium carrying the cloned bet genes of E. coli expressed all three Bet activities, whereas the parental strain lacks these activities. These findings show that we have cloned the structural bet genes of E. coli, rather than a regulatory region.

The gene encoding choline dehydrogenase was termed betA (Styrvold et al., 1986). The smallest chromosomal fragment isolated which expressed this activity was 3.3 kb, and was the most distal part of the bet region relative to the lac operon. The molecular mass of the choline dehydrogenase of E. coli is not known, but the corresponding mammalian mitochondrial enzyme consists of one polypeptide chain of about 74 kDa (Tsuge et al., 1980), requiring a coding region of 2 kb. Correspondingly, the smallest DNA fragment isolated which expressed only the betaine aldehyde dehydrogenase was 3.7 kb and constituted the middle part of the bet region. This dehydrogenase comprised a tetramer of four identical subunits, each with a molecular mass of about 55 kDa as judged by SDS-PAGE; thus, the enzyme required a coding region of about 1.5 kb (P. Falkenberg & A. R. Ström, unpublished data). To our knowledge, there are no reports on mutants which are specifically blocked in the synthesis of the betaine aldehyde dehydrogenase. On the basis of the present cloning analysis, we name the structural gene for this enzyme betB.

The cloning analysis also indicated that the gene(s) governing the osmotically regulated high-affinity transport system for choline was located clockwise of the betA,B genes (i.e. proximal to the lac region). We do not know whether this system is driven by a protonmotive force (such systems are often coded for by one gene), or is a binding-protein-dependent system requiring several genes such as the ProU system for osmotically regulated glycine betaine transport (Gowrishankar et al., 1986; Higgins et al., 1987; May et al., 1986). However, for simple identification of the high-affinity choline transport system, we suggest the notation betT for this gene or operon. Thus, the order of the named bet genes or gene systems is, in a clockwise direction, betA,B,T. In a preliminary account of this work it was thought that the betT was located on the other side of the betA,B genes (Strøm et al., 1986).

Amino acid starvation and other conditions which prevent protein synthesis seem to be the only physiological conditions known to increase the copy number of plasmids (Adams & Hatfield, 1984). Thus, assuming that osmotic stress did not increase the copy number of the plasmids used in this study, our data show that the cloned betA,B,T genes were osmotically regulated. This finding is in agreement with our previous data for strain K10 (Landfald & Strøm, 1986; Styrvold et al., 1986), and supports the contention that the cloned genes were transcribed from their native promoters.

The present data indicate that there must be at least three promoters in the bet region which are activated by growth in minimal medium of elevated osmotic strength. From the previous biochemical analysis of K10, we suggested that the genes for the BetA and the BetB dehydrogenases are located in the same operon or share a common regulatory system, whereas the genes for the BetT transport system represent a separate transcriptional unit (Landfald &
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CAIRNEY, J., BOOTH, I. R.


E. coli enteric bacteria harbouring the mutant allele (Csonka, 1981) transfer of naturally occurring osmotic tolerance genes between species. It has, however, been shown that an increased dosage of the indigenous choline therefore did not accumulate glycine betaine when grown under osmotic stress in the presence of glycine betaine (Gowrishankar, 1986), and that a mutation in the proU region of E. coli leading to proline overproduction gives increased osmotic tolerance to enteric bacteria harbouring the mutant allele (Csonka, 1981; Jakowec et al., 1985).

Our data show that S. typhimurium carrying a broad-host-range plasmid with the bet genes of E. coli was more osmotically tolerant in the presence of choline than the parental strain. To our knowledge this is the first example of genetic engineering of osmotic tolerance of bacteria by transfer of naturally occurring osmotic tolerance genes between species. It has, however, been shown that an increased dosage of the indigenous proU gene increases the osmotic tolerance of E. coli in the presence of glycine betaine (Gowrishankar et al., 1986), and that a mutation in the proAB region of E. coli leading to proline overproduction gives increased osmotic tolerance to enteric bacteria harbouring the mutant allele (Csonka, 1981; Jakowec et al., 1985).

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HIGGINS, C. F., SUTHERLAND, L., CAIRNEY, J. &


