Identification of the Genes for the Lactose-specific Components of the Phosphotransferase System in the \( \text{lac} \) Operon of \( \text{Staphylococcus aureus} \)*

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The nucleotide and deduced amino acid sequences of the \( \text{lacE} \) and \( \text{lacF} \) genes, which code for the lactose-specific Enzyme II and Enzyme III of the \( \text{Staphylococcus aureus} \) phosphotransferase system, are presented. The primary translation products consist of a hydrophobic protein of 572 amino acids (\( M_r = 62,688 \)) and a polypeptide of 103 amino acids (\( M_r = 11,372 \)), respectively. The assignment of \( \text{lacF} \) as the gene for Enzyme III is based upon the known amino acid sequence of the protein. The identity of \( \text{lacE} \) as encoding Enzyme II was based upon immunoreactivity of the cloned gene product with antibodies raised against purified Enzyme II* from \( \text{S. aureus} \) and an assay of biological function of the protein expressed in \( \text{Escherichia coli} \). The order of the known genes of the \( \text{S. aureus} \) lac operon is \( \text{lacF}-\text{lacE}-\text{lacG} \), the latter encoding phospho-\( \beta \)-galactosidase.

Metabolism of lactose by the Gram-positive bacterium \( \text{Staphylococcus aureus} \) is initiated by the uptake of the carbohydrate by the PTS1 (1–3). Two of the four proteins which comprise this transport system, \( \text{EII'} \) and \( \text{EIII'} \), are specific for \( \beta \)-galactosides. The intracellular lactose phosphate is then cleaved into glucose and galactose-\( \beta \)-phosphate in a reaction catalyzed by phospho-\( \beta \)-galactosidase (4). The lactose-specific protein products have been characterized. Phospho-\( \beta \)-galactosidase is a monomeric protein with a molecular weight of 54,557 (4, 5). \( \text{EIIm} \) is an integral membrane protein with an apparent molecular weight of 55,000, as estimated by SDS-polyacrylamide gel electrophoresis (6). \( \text{EIIf} \) consists of three identical subunits of molecular weight 12,000 (7, 8).

\( \text{EIIm} \), \( \text{EIIf} \), and phospho-\( \beta \)-galactosidase are inducible proteins, and their genes have been shown to be linked on the staphylococcal chromosome (9, 10). We have cloned the phospho-\( \beta \)-galactosidase gene (\( \text{lacG} \) in \( \text{Escherichia coli} \)) and have determined its nucleotide sequence (11). This analysis indicated that \( \text{lacG} \) is the terminal determinant of a polycistrionic operon. We present here the nucleotide sequence of the gene for \( \text{EIIm} \) (\( \text{lacE} \)) and \( \text{EIIf} \) (\( \text{lacF} \)). We present evidence that these cloned gene products, expressed in \( \text{E. coli} \), are indeed \( \text{EIIm} \) and \( \text{EIIf} \). This is the first report of the primary sequence of an \( \text{EII} \) and \( \text{EIII} \) of the PTS from a Gram-positive bacterium. The amino acid sequence of \( \text{EIIf} \), deduced from the nucleotide sequence, displays structural similarities with \( \text{EIIf} \) and \( \text{EIIf} \) of \( \text{E. coli} \) (12, 13).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Bacteriophage—** \( \text{E. coli JM101} \) was used as a host for the sequencing coliphage vectors M13mp10 and M13mp11 (14). Plasmids pFB34 and pFB40 (5) were maintained in \( \text{E. coli LE392} \) (15). \( \text{E. coli CMK} \) carries the cloned \( \text{EIII'} \) from \( \text{Lactobacillus casei} \).\(^2\) The \( \text{S. aureus} \) strains used were wild-type strain S5601 and strain S305A, constitutive for the lactose-specific components of the PTS (16).

**Determination of Nucleotide Sequence—** DNA manipulations were as previously reported (5). DNA sequencing was conducted by the dideoxy chain termination method (17). Computer analysis on the sequence was carried out with “Sequid” (18), a software package provided by D. Rhoades and D. Roufa (Kansas State University). Hydropathy analysis of the protein product of \( \text{lacE} \) was described by Kyte and Doolittle (19).

**Preparation of Membranes—** \( \text{S. aureus} \) strains S5601 and S6601 (not induced for the lac operon) were cultivated, harvested, and disrupted as described previously (20). The crude membranes were sedimented at 13,000 × \( g \) overnight or at 170,000 × \( g \) for 4 h. \( \text{E. coli LE392} \) harboring the plasmids pFB34 or pFB40 (5) were grown overnight in Luria broth containing 50 mg/liter ampicillin. 15 g of cells from 9 liters of culture were suspended in 40 ml of buffer A (0.05 m Tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 7.5) and disrupted by sonication. Membranes were sedimented as described above and washed with buffer to remove residual basal level \( \beta \)-galactosidase, which interfered with the \( \text{S. aureus} \) mutant complementation assay (16). The supernatant was chromatographed on a Sephadex G-75 column (70 × 5 cm) to remove \( \text{E. coli} \) \( \beta \)-galactosidase. This step was necessary to assay for \( \text{EIIm} \) of \( \text{S. aureus} \).

**Purification of Denatured EIIf of S. aureus—** One g of crude membranes were suspended in 10 ml of buffer A. The pH of the suspension was adjusted to 12.4 by the addition of 1 ml of 1 m NaOH. \( \text{EIIf} \) was then sedimented at 170,000 × \( g \) for 3 h at 4 °C. Up to 0.2 g of the sediment were homogenized in 2 ml of buffer B (0.08 m Tris/glycine, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM lactose, pH 9.3) (6). One ml of S305A “dansylated membranes” and 1.5 ml of 3 × sample buffer* were added and applied to preparative SDS-polyacrylamide gel electrophoresis. For the preparation of \( \text{EIIm} \), 10% acryl-
amide gels (150 × 100 × 5 mm) were run; analytical gels were 1-mm thick. Electrophoresis was performed at 40 V, constant voltage, for 16–19 h at room temperature. The proteins were visualized by UV illumination at 366 nm, excised, homogenized, and subjected to electrophoresis in an apparatus purchased from Orpogen (Heidelberg) for 16 h at 30 V and room temperature. The elution buffer was 0.1% SDS, 0.05 M NaHCO₃; the soaking buffer was 2% SDS, 0.4 M NaHCO₃. Excessive SDS and salt were removed by dialysis against 5 liters of 0.02 M sodium phosphate buffer, 0.02% SDS, pH 6.8, overnight. The protein solution was concentrated in a vacuum centrifuge. The protein yield from 580 mg of NaOH-extracted membranes was 1.6 mg of EIIIC as determined by a modified Lowry procedure.

To produce the internal fluorescent protein standard, 15 mg (wet weight) of NaOH-extracted membranes were dissolved in 0.85 ml of Tris/glycine/SDS buffer (0.1 M, pH 9) and 0.15 ml of 20% SDS. Forty pl of a 10% solution of dansyl chloride in acetonitrile was added with vigorous shaking. The mixture was incubated at 70 °C for 2 min; the reaction was stopped by adding 20 μl of β-mercaptoethanol and agitating for 2 min at 70 °C. Low molecular weight products were removed by passage through a Pasteur pipette filled with Sephadex G-25 equilibrated with gel sample buffer. The separation was followed under UV light. The mixture could be stored frozen (21).

Production of Anti-EIIIC Antibodies—540 pg of electroeluted concentrated EIIIC were hemogenized with complete Freund's adjuvant and injected intramuscularly into a rabbit. Thirty-five days later, a subcutaneous booster injection with the same amount of EIIIC in Freund's complete adjuvant was made. A second booster injection was performed at day 63 with 180 pg of protein in incomplete Freund's adjuvant. The rabbit was drugged with ether at day 72 and exsanguinated, which yielded 200 ml of blood.

Western Blot Analysis—Western blotting was carried out according to the Bio-Rad Immun-Blot protein A horseradish peroxidase conjugate manual. Antiserum was applied in a dilution of 1:60. The proteins were transferred from an analytical SDS-polyacrylamide gel to a nitrocellulose sheet (Schleicher and Schuell, 0.45 μm). The transfer was performed at 4 °C for 30 min at 10 V/cm and then for 1 h at 15 V/cm. The transfer buffer had the following composition: 20% methanol, 3 g of Tris, 14.4 g of glycine, and 1 g of SDS/1000 ml.

Assay of EIIIC Activity—A mutant complementation assay with an extract of strain S305A, defective in EIIIC, was used as previously described (22).

Phosphorylation of EIIIC of S. aureus with [γ-32P]ATP—Phosphorylation of EIIIC was performed in 44 μl of 50 mM NH₄HCO₃ with the following components: 500 nmol of MgCl₂, 5 μg of Enzyme I from Streptococcus faecalis (22), 2 μg of HP from S. faecalis (24), 2 μg of EIIIC of S. aureus (7), 100 μg of membrane protein; EIIIC, 0.1 μCi of [γ-32P]ATP (25). To identify labeled proteins on an SDS gel, reaction mixtures without EII, EIII, and HP were run. To demonstrate strict PEP dependence, 5 nmol of ATP were included; specific dephosphorylation of EIIIC was achieved by the addition of 5 nmol of isopropyl β-D-thiogalactopyranoside to the complete phosphorylation mixture.
being an integral membrane protein. The average hydropathy values for EII'' and EIIbg of E. coli are plotted in the sequence.

Hydropathy analysis: It is presented in Table I. Consistent with the residues 1-326 being separated from the approximately 370 base pairs from the EcoRI site of the pFB34 insert and extends through the initiation codon of lacC. The sequence given is contiguous; the lower part sequences for clarity.

Hydropathy analysis of the lacE-deduced amino acid sequence. Hydropathy was calculated according to Kyte and Doolittle (20). Seven amino acids were averaged at a time. Values for residues 1-326 are presented in the upper graph, and those for residues 327-572 are plotted in the lower part of the figure.

The deduced amino acid composition of the lacF and lacE genes is presented in Table I. Consistent with the hydropathy analysis, the lacE product contains a high percentage of the nonpolar amino acids alanine, phenylalanine, glycine, isoleucine, leucine, and valine. The lacE protein thus has features suggesting it is EII''', although it is larger than the reported size of this protein. Disparity may be due to protein processing or anomalous migration of this hydrophobic protein in SDS gels. It is of interest to note that this protein was not detected by E. coli strains harboring the lac operon, suggesting that the lac operon is expressed from a different promoter in these strains.

Identification of the lacE Gene Product as EII'. SDS-polyacrylamide gel electrophoresis of NaOH-extracted membranes of the lacE-containing S. aureus strain S305A showed a prominent 48-kDa protein band, which was absent in the wild-type strain S5601 not induced for lactose metabolism. This is smaller than the size previously determined for EII' (6). The discrepancy may be a
function of the different gel systems used. Purified EIIaw was prepared and used to immunize rabbits. The resulting antiserum was used to probe lysates of E. coli carrying plasmid pFB34. As shown in Fig. 4A, the antibodies recognized a 48-kDa species in the lysate from pFB34-carrying cells, but not with a control E. coli lacking this plasmid. The protein produced in E. coli comigrates with purified EIIaw from S. aureus (Fig. 4B). The same immunoreactive protein was observed with LE392 cells harboring pFB40 (5), a deletion subclone of pFR34 which carries the entire lacE coding sequence (data not shown).

Biological activity of the product of the cloned lacE gene was measured using a mutant complementation assay. Values obtained with E. coli LE392 (pFB34) and S. aureus S305A were 15 and 60 nmol of o-nitrophenyl-β-D-galactopyranoside 6-phosphate/min/mg of membrane protein, respectively. The biological activity and immunoreactivity thus establish the lacE gene as the determinant of EIIaw of S. aureus.

Codon Usage Analysis—Work with E. coli has established that there is a correlation between expression level of a gene and its codon usage pattern. The more highly expressed genes display a nonrandom pattern of codon usage, utilizing a restricted set of codons which are recognized by major species of isoacceptor tRNAs, while genes expressed at low levels have a more random pattern of codon usage (28, 29). This trend may also hold true for the Gram-positive bacterium Bacillus subtilis (30). The codon usage patterns for the three sequenced genes of the S. aureus lac operon are presented in Table II. The codon usage is biased toward A- or U-rich codons as expected for genes of the relatively low G+C content of S. aureus DNA (32–35 mol %). However, some codons such as AUA (isoleucine), which are rarely used in highly expressed genes in E. coli, are infrequently used in these S. aureus messages despite its A+U-rich nature. This bias against AUA is not found in all S. aureus genes (11).

Organization of the S. aureus lac Operon and Intercistronic Regions—A stem-loop structure resembling a transcription terminator is positioned immediately 3' to lacG suggesting this is the terminal gene of this transcriptional unit (11). The gene order of the operon is lacF-lacE-lacG. Additional significant open reading frame(s) can be found upstream of lacF.

| Amino acid | Count | Mol % | Count | Mol % |
|------------|-------|-------|-------|-------|
| Ala        | 16    | 15.53 | 58    | 10.14 |
| Cys        | 1     | 0.97  | 3     | 0.52  |
| Asp        | 7     | 6.80  | 23    | 4.02  |
| Glu        | 9     | 8.74  | 25    | 4.37  |
| Phe        | 5     | 4.85  | 46    | 8.04  |
| Gly        | 7     | 6.80  | 43    | 7.52  |
| His        | 4     | 3.88  | 4     | 0.70  |
| Ile        | 5     | 4.85  | 54    | 9.44  |
| Lys        | 6     | 5.83  | 37    | 6.47  |
| Leu        | 13    | 12.62 | 54    | 9.44  |
| Met        | 1     | 4.85  | 19    | 3.32  |
| Asn        | 3     | 2.91  | 27    | 4.72  |
| Pro        | 0     | 0.00  | 25    | 4.37  |
| Gln        | 1     | 1.94  | 46    | 8.04  |
| Arg        | 3     | 2.91  | 31    | 5.42  |
| Ser        | 3     | 2.91  | 32    | 5.99  |
| Thr        | 5     | 4.85  | 47    | 7.34  |
| Val        | 3     | 2.91  | 12    | 2.12  |
| Trp        | 0     | 0.00  | 7     | 1.22  |
| Tyr        | 2     | 1.94  | 15    | 2.62  |
| Total      | 103   | 572   |       |       |

**Table 1**

| Amino acid | Count | Mol % |
|------------|-------|-------|
| Ala        | 16    | 15.53 |
| Cys        | 1     | 0.97  |
| Asp        | 7     | 6.80  |
| Glu        | 9     | 8.74  |
| Phe        | 5     | 4.85  |
| Gly        | 7     | 6.80  |
| His        | 4     | 3.88  |
| Ile        | 5     | 4.85  |
| Lys        | 6     | 5.83  |
| Leu        | 13    | 12.62 |
| Met        | 1     | 4.85  |
| Asn        | 3     | 2.91  |
| Pro        | 0     | 0.00  |
| Gln        | 5     | 4.85  |
| Arg        | 4     | 3.88  |
| Ser        | 3     | 2.91  |
| Thr        | 5     | 4.85  |
| Val        | 3     | 2.91  |
| Trp        | 0     | 0.00  |
| Tyr        | 2     | 1.94  |
| Total      | 103   | 572   |
TABLE II

| Amino Acid | Codon  | lacF | lacE | lacG |
|------------|--------|------|------|------|
| Phe        | UUU    | 5    | 28   | 15   |
| Phe        | UUC    | 0    | 18   | 11   |
| Leu        | UUA    | 9    | 33   | 21   |
| Leu        | UUG    | 1    | 8    | 3    |
| Leu        | CUU    | 0    | 8    | 1    |
| Leu        | CUC    | 0    | 0    | 0    |
| Leu        | CUA    | 0    | 5    | 1    |
| Leu        | CUG    | 3    | 0    | 0    |
| Ile        | AUU    | 5    | 39   | 17   |
| Ile        | AUC    | 0    | 13   | 11   |
| Ile        | AUA    | 0    | 2    | 1    |
| Met        | AUG    | 5    | 19   | 9    |
| Val        | GUU    | 1    | 11   | 12   |
| Val        | GUC    | 1    | 6    | 3    |
| Val        | GUA    | 1    | 18   | 10   |
| Val        | GUG    | 0    | 7    | 5    |
| Ser        | UCU    | 1    | 6    | 7    |
| Ser        | UCC    | 0    | 0    | 0    |
| Ser        | UCA    | 0    | 15   | 5    |
| Ser        | UCG    | 0    | 0    | 0    |
| Pro        | CCU    | 0    | 3    | 9    |
| Pro        | CCC    | 0    | 0    | 0    |
| Pro        | CCA    | 0    | 17   | 9    |
| Pro        | CCG    | 0    | 5    | 1    |
| Thr        | ACU    | 0    | 4    | 5    |
| Thr        | ACC    | 0    | 0    | 0    |
| Thr        | ACA    | 4    | 23   | 13   |
| Thr        | ACG    | 1    | 5    | 5    |
| Ala        | GCU    | 3    | 9    | 5    |
| Ala        | GCC    | 0    | 8    | 3    |
| Ala        | GCA    | 10   | 29   | 17   |
| Ala        | GCG    | 3    | 12   | 3    |

**lacF**

| Codon  | lacF | lacE | lacG |
|--------|------|------|------|
| UUCCUCC |      |      |      |

**lacE**

| Codon  | lacF | lacE | lacG |
|--------|------|------|------|
| AUAAAAGGUGUUAAUGGAAUGGAU |      |      |      |

**lacG**

| Codon  | lacF | lacE | lacG |
|--------|------|------|------|
| AUUAAGGAUGGGAUGAUGGAUACAGA |      |      |      |

Fig. 5. Intercistronic regions of the lac operon. Given are the sequences corresponding to the mRNA of the junction between the upstream open reading frame and lacF, lacE and lacG. Immediately above each sequence is given the complementary sequence from the 3'-terminus of the B. subtilis 16 S rRNA (written 3' → 5'). Termination and initiation codons are underlined. The predicted free energy of base pairing (ΔG) of the ribosome binding sites with 16 S rRNA was calculated according to the rules of Tinoco et al. (31). Spacer distances were calculated as described (35).

Fig. 6. Phosphorylation of EII₃ of S. aureus S305A with [³²P]PEP. Autoradiograph of proteins resolved on an SDS-10% polyacrylamide gel. Additions to the reaction mixture were: lane 1, 0.1 μCi of [³²P]PEP and 5 μg of Enzyme I; lane 2, 0.1 μCi of [³²P]PEP, 5 μg of Enzyme I, and 2 μg of HPr; lane 3, 0.1 μCi of [³²P]PEP, 5 μg of Enzyme I, 2 μg of HPr, and 2 μg of EII₃; lane 4, 0.1 μCi of [³²P]PEP, 5 μg of Enzyme I, 2 μg of HPr, 2 μg of EII₃, and 5 nmol of isopropyl β-D-thiogalactopyranoside; lane 5, 0.1 μCi of [³²P]PEP, 5 μg of Enzyme I, 2 μg of HPr, 2 μg of EII₃, and EII₃ (100 μg of membrane protein), and 5 nmol of isopropyl β-D-thiogalactopyranoside) which is a substrate for the lactose PTS. The phosphorylated residue is most likely histidine (35, 36). The reduced amino acid sequence of the lacE gene product revealed only four histidine residues. This feature of the S. aureus EII₃ should greatly facilitate the localization of the active site histidine by means of site-directed mutagenesis.

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The number tabulated is the total number for each codon in each gene.

Amino acid occurs without the requirement of the ribosome binding sequence. Two additional ATG codons are found 4 and 7 nucleotides 3' to the first ATG. All three ATG codons are in the same reading frame; use of either of the latter two would omit two (Met-Thr) or three (Met-Thr-Met) amino acids from the amino terminus of the protein. The third initiation codon, 10 nucleotides from the ribosome site, has the more favorable spacing.

There is little intercistronic space in this operon. The ribosome binding site sequence for lacE is within the terminal coding sequence of lacF, that of lacG overlaps by one nucleotide pair the ochre termination codon of lacG, while the ribosome binding sequence of lacF is 5 base pairs downstream of the ochre codon of the preceding open reading frame. The buried nature of the ribosome binding site for lacE raises the possibility that translating ribosomes may initiate at the first AUG codon, while a ribosome initiating translation at this ribosome binding site may require a greater spacing and thus initiate at the third AUG codon. This possibility will be resolved when the N-terminal amino acid sequence of the protein becomes available.

Phosphorylation of EII₃ of S. aureus—As shown in Fig. 6, EII₃ of S. aureus forms a phosphorylated intermediate (lane 5) which can be specifically dephosphorylated with the addition of a compound (such as isopropyl β-D-thiogalactopyranoside) which is a substrate for the lactose PTS. The phosphorylated residue is most likely histidine (35, 36). The deduced amino acid sequence of the lacE gene product revealed only four histidine residues. This feature of the S. aureus EII₃ should greatly facilitate the localization of the active site histidine by means of site-directed mutagenesis.

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