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Metabolism of Lactose by *Staphylococcus aureus* and Its Genetic Basis

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The metabolism of lactose was found to be controlled by three genes: a gene for the synthesis of a β-galactosidase attacking only phosphorylated galactosides; a gene for a protein permitting concentration of phosphorylated galactosides which probably acts by transferring phosphates to them; and a gene regulating the first two structural genes. The three genes are closely linked and may have the same order as in *Escherichia coli*. Galactose-6-phosphate was found to be a better inducer of lactose utilization than is galactose or any other inducer. The inhibition of induction by isopropylthiogalactoside was found to occur at the level of the protein permitting the concentration of galactoside phosphates.

The reports of Creaser (2) and McClatchy and Rosenblum (11) indicate that the metabolism, the regulatory control of the metabolism, and the genetic arrangement of the genes of *Staphylococcus aureus* involved in lactose utilization may be similar to those found in *Escherichia coli*. However, some differences were noted: (i) the β-galactosidase appeared very labile, and impossible of separation from whole staphylococcal cells; (ii) the gratuitous inducer, isopropylthiogalactoside (IPTG), employed in *E. coli* studies, was an inhibitor of induction in *S. aureus*; (iii) although genetic fine structural analysis was not attempted, McClatchy and Rosenblum found that a gene for constitutive synthesis of β-galactosidase and for lactose uptake was not linked to either the gene for β-galactosidase or the permease gene. They also found recombination between the β-galactosidase and the permease genes.

Recently, we presented evidence (6) that the apparent in vitro lability of the staphylococcal β-galactosidase was an artifact; the proper substrates of this enzyme are the phosphates of lactose, or of *o*-nitrophenyl-galactoside (ONPG), since the enzyme has no activity on the nonphosphorylated compounds. It has also been shown that staphylococci accumulate carbohydrates by phosphorylating them, a process which can be destroyed by the pleiotropic *car* mutation (4, 5; Hengstenberg and Morse, *Carbohydrate Research, in press*; Hengstenberg and Morse, J. Biol. Chem., *in press*). The finding of a new β-galactosidase prompted further investigation of lactose utilization by *S. aureus*.

MATERIALS AND METHODS

The cultures employed were derivatives of *S. aureus* NCTC 8511, the propagating strain for typing phage 53 (NCTC 8406), which was used previously (3, 12). A list of the cultures, with genotypes, is given in Table 1. Most of the mutants employed were obtained after treatment with *N,N'*-nitrosoguanidine (5 μg/ml; 3 hr in phosphate buffer, *p*H 6.2) and plating on indicator agar containing the appropriate carbohydrate(s).

Liquid cultures were made in 2% peptone (Difco), and various solid media were employed: (i) 1% nutrient agar or 1% tryptone-agar plates; (ii) the cholate-neutral red-indicator agar (13) and the Korman-Berman medium (9) for detecting carbohydrate utilization; and (iii), for the selection of transductants, the medium of Murphey and Rosenblum (14) containing the appropriate carbohydrates.

ONPG and IPTG were obtained from Mann Research Laboratories; galactose (glucose-free), galactose-6-phosphate (Gal-6-P), D-fucose, and 2-deoxygalactose, from Sigma Chemical Co.; lactose, from Pfanstiehl Laboratories; ^{14}C-labeled lactose (0.5 mc/mmole), from Calbiochem.

Samples containing radioactive lactose were counted in a Beckman CPM-100 Scintillation Counter employing toluene and 2,5-diphenyloxazole (Packard).

The lysis of staphylococcal suspensions was accomplished with lysostaphin (Mead, Johnson and Co.), generously supplied by Peter Tavormina.

RESULTS

Mutations affecting lactose utilization. McClatchy and Rosenblum (11) reported three mutations in *S. aureus* affecting lactose utilization:

TABLE 1. *Cultures of Staphylococcus aureus NCTC 8511 employed*

Culture	Genotype	Phenotype
5601	*sm*^{r} *53*$^+$	Streptomycin-resistant, lysogenic for phage 53
203A, 306A, 307A, 322Q	*sm*^{r} *53*$^+$ *r*$^-$	As 5601, produce β-galactosidase constitutively. Independent isolations
305A	*sm*^{r} *53*$^+$ *suc*$^-$ *r*$^-$	As 5601, also sucraseless, produced β-galactosidase constitutively
420H	*sm*^{r} *53*$^+$ *cnc*$^-$	As 5601, also Lac$^-$ Gal$^-$
420K	*sm*^{r} *53*$^+$ *z*$^-$	As 5601, also β-galactosidaseless
714B	*sm*^{r} *53*$^+$ *suc*$^-$ *r*$^-$ *cnc*$^-$	As 305A, also Lac$^-$ Gal$^-$
714K	*sm*^{r} *53*$^+$ *suc*$^-$ *r*$^-$ *cnc*$^-$	As 714B
744A	*sm*^{r} *53*$^+$ *z*$^-$ *cnc*$^-$	As 5601, also β-galactosidaseless, Lac$^-$ Gal$^-$
756B	*sm*^{r} *53*$^+$ *z*$^-$ *cnc*$^-$	As 744A

The accumulation of lactose probably occurs through a scheme similar to that described in *E. coli* (10) involving phosphoenolpyruvate; HPr, a heat-stable protein; and two enzymes, E I and E II. The *cnc* mutants are defective in the E II for lactose utilization, whereas the car mutants described previously (3, 4, 5) are defective in E I or HPr.

(i) a mutation causing loss of ability to utilize lactose, and of the ability of whole cells to hydrolyze ONPG; (ii) a mutation which causes loss of the ability of whole cells to take up lactose-*1*-^{14}*C*; (iii) a regulator gene change which produced constitutive formation of i and ii. We confirm these observations and add that mutants under ii are pleiotropic and are unable to utilize galactose in addition (two of McClatchy and Rosenblum's strains, *lac-1*$^-$ and *lac-2*$^-$, presumed permease mutants received from Dr. Rosenblum, were also examined in addition to our own mutants and found to have this phenotype). The double mutants are unable to concentrate radioactive lactose but are not impermeable to it (Table 2). In comparison with *z* mutants (6), the *cnc* mutants are unable to form lactose phosphate. Since the mutation affects ability to *concentrate* rather than permeability, we designate this gene "*cnc*."

We have isolated several mutants constitutive for the uptake and hydrolysis of β-galactosides according to the procedure of McClatchy and Rosenblum but, like them, cannot determine whether they represent mutations comparable to *i*$^-$ or o^c mutations in *E. coli.*

Induction of lactose metabolism. The induction of lactose metabolism was studied previously (2, 11) and the most efficient inducer found to be galactose. In addition, IPTG was found (2) to be a competitive inhibitor of induction. A number of inducers were tested with and without IPTG on the wild-type strain for inducing ability (Table 3). Gal-6-P was found to induce approximately eightfold higher enzyme activity than galactose. With the exception of the case of lactose, the presence of 10^{-3} M IPTG inhibited induction greatly.

The importance of Gal-6-P as an inducer is

TABLE 2. *Permeability of the cnc mutants to lactose-1-*^{14}*C*[a]

Preparation	Activity	Counting error
	counts/min	%
Control sample	2,794	0.5
Supernatant sample	3,281	0.5

[a] To a 3-ml suspension of the *cnc* mutant 714K, which contained 1.5 ml of cells, was added 0.05 μc of lactose-*1*-^{14}C, and the suspension was incubated at 37 C for 15 min. At the same time and in an identical manner, the same amount of radioactive lactose was added to a control 3-ml volume containing no cells. The tubes were centrifuged in the cold; 0.05-ml samples were removed from the tube with no cells and from the supernatant fluid of the tube with cells and counted in a scintillation counter. If the *cnc* cells had not been freely permeable to lactose-*1*-^{14}C, the number of counts in the supernatant sample would be twice that of the sample from the control.

indicated by a study of induction in the *cnc*$^-$ mutants which are unable to phosphorylate lactose on the 6 position of the galactose moiety. Galactose has little or no inducing activity in *cnc*$^-$ mutants but Gal-6-P does (Table 4), almost as much as galactose produces in the wild type. These observations suggest that the *cnc*$^-$ mutants, unable to utilize both lactose and galactose, are unable to phosphorylate galactose as well as lactose. Chromatographic analysis of *cnc*$^-$ suspensions exposed to radioactive galactose failed to show the formation of derivatives, whereas the wild type does produce a derivative that corresponds to galactose phosphate.

Previously (Table 3), IPTG was found to inhibit induction of the wild type by Gal-6-P. The site of inhibition is revealed by the study of in-

TABLE 3. *Induction of lactose utilization in the wild type and its inhibition by isopropylthiogalactoside (IPTG)*[a]

Inducer	10^{-3} M IPTG	Enzyme formed[b]
Gal-6-P	−	440.0
	+	88.8
Galactose	−	59.2
	+	31.0
Lactose	−	83.3
	+	83.3
D-Fucose	−	11.1
	+	5.6
2-Deoxygalactose	−	7.4
	+	3.7
ONPG	−	46.3
	+	3.7

[a] The wild-type culture 5601 was used. Induction was in broth cultures (1 ml, optical density value of 1.4) containing the inducers at 10^{-2} M concentration with and without 10^{-3} M IPTG for 3 hr, at which time 0.01 ml of a lysostaphin (1 mg/ml) solution was added to lyse the cells. The amount of enzyme formed was assayed in 0.2-ml samples, by using ONPG-phosphate (0.2 ml of a 12 μmoles/ml stock) and 0.6 ml of tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 7.6, containing 0.05 M NaCl and 0.05 M $MgCl_2$) in a Beckman DB spectrophotometer with a Photovolt recording attachment.

[b] Expressed as micromoles of ONPG per minute per gram (dry weight) of cells.

TABLE 4. *Induction of cnc mutants by galactose and galactose-6-phosphate (Gal-6-P)*[a]

Induction time	Galactose induction	Gal-6-P induction
min		
90	7.4	7.4
120	7.4	14.8
150	7.4	22.2
180	7.4	41.2

[a] The cnc^- strain 420H was employed and the procedure was essentially that of Table 2 except that samples were taken with time. Inducer concentration, 3.3×10^{-2} M. The results are expressed as micromoles of ONPG released from ONPG-phosphate per minute per gram (dry weight) of cells. The values shown for galactose probably represent the basal level of the culture.

TABLE 5. *Lack of inhibition by IPTG of the induction by Gal-6-P in the cnc mutant*[a]

Induction time	Gal-6-P induction	Gal-6-P + IPTG induction
min		
90	7.4	6.7
120	11.1	12.3
150	18.5	18.5
180	29.5	28.3

[a] Results expressed as in Table 4; procedures as in Tables 2 and 3. Gal-6-P concentration, 10^{-2} M; IPTG, 10^{-3} M.

hibition of Gal-6-P induction by IPTG in *cnc* mutants. Table 5 shows that IPTG has no inhibitory activity and therefore the site of inhibition is the functional product of the *cnc* gene.

Additional clarification of the induction process was obtained from the measurement of the induced state by the uptake of radioactive lactose, employing a mutant defective in β-galactosidase formation. Under these circumstances it is found that galactose induces lactose uptake, but lactose does not, a finding that indicates that it is the formation of Gal-6-P from lactose phosphate that is necessary for induction by lactose. The β-galactosidaseless mutant is unable to split lactose phosphate to Gal-6-P and glucose, although it is able to form and accumulate lactose phosphate if previously induced with galactose or Gal-6-P.

Genetic analysis of mutants. McClatchy and Rosenblum found in transductional analysis that there was recombination to yield wild type between their permease mutants (cnc^-) and a mutant defective in its ability to utilize lactose or to hydrolyze ONPG (z^-). In addition, they found no cotransduction of the constitutive character with transductions of the permease or of the β-galactosidase activity. We have examined the genetic linkage in strain 8511 and its derivatives. Utilizing five independent constitutive donors ($r^- z^+ cnc^+$), transductions were made to a double mutant ($r^+ z^- cnc^-$) obtained after sequential exposure to nitrosoguanidine, firstly to obtain a β-galactosidaseless (z^-), and secondarily to obtain the cnc^- phenotype. Selection of transductants was made on medium containing lactose which requires that each transductant receive both z^+ and cnc^+. The fact that transductants were obtained indicates close linkage of *z* to *cnc*. All five independent regulator gene mutants were found linked to the structural genes (7% recombination). The closeness of the linkage of *z* to *cnc* was tested directly by reciprocal transductions between z^- and cnc^- mutants which, in a small number of transductions, indicated less than 1% recombination between them. A more extensive test was made (Table 6, experiment 3) by selection for cnc^+ on galactose-containing medium. This test indicated less than 1% recombination between *z* and *cnc*, and 9.5% between *r* and *z*.

TABLE 6. *Genetic analysis of lactose mutations*

Expt	Donor	Genotype	Recipient	Genotype	Transductants			Cotransduction
					$r^- z^+ cnc^+$	$r^+ z^+ cnc^+$	$r^+ z^- cnc^+$	
								%
1[a]	305A	$r^- z^+ cnc^+$	744A	$r^+ z^- cnc^-$	95	5	—	95
	203A	$r^- z^+ cnc^+$	744A	$r^+ z^- cnc^-$	23	2	—	92
	306A	$r^- z^+ cnc^+$	744A	$r^+ z^- cnc^-$	24	1	—	96
	307A	$r^- z^+ cnc^+$	744A	$r^+ z^- cnc^-$	21	4	—	88
	322Q	$r^- z^+ cnc^+$	744A	$r^+ z^- cnc^-$	23	2	—	92
2[b]	420K	$r^+ z^- cnc^+$	420H	$r^+ z^+ cnc^-$	—	0	—	—
	5601	$r^+ z^+ cnc^+$	420H	$r^+ z^+ cnc^-$	—	59	—	—
	420H	$r^+ z^+ cnc^-$	420K	$r^+ z^- cnc^+$	—	0	—	—
	5601	$r^+ z^+ cnc^+$	420K	$r^+ z^- cnc^+$	—	168	—	—
3[c]	306A	$r^- z^+ cnc^+$	756B	$r^+ z^- cnc^-$	683	72	1	*r— z*, 90 .5 *z — cnc*, 99.9

[a] Five independent isolations of constitutive mutants. Selection was on lactose, and required both z^+ and cnc^+. The fact that transductants were obtained indicates close linkage of *z* and *cnc*. Average cotransduction of *r* and *z* was 93%.

[b] Close linkage is indicated by the failure to obtain wild-type recombinants among mutants. Selection of transductants was on lactose-containing media.

[c] Selection of transductants on galactose-containing media which requires only cnc^+, and *r* and *z* are unselected. Only one recombinant between *z* and *cnc* was found in the 756 transductants tested, confirmation of the failure to detect recombinants directly in experiment 2.

Biochemical studies. When extracts of staphylococci are supplemented with phosphoenolpyruvate and ONPG, they form ONPG-phosphate, which in the presence of the staphylococcal β-galactosidase is hydrolyzed to yield ONP (6, 8). More recent experiments permit fractionation of the system, and the study of specific mutations, in mixed extracts. These complementation studies will be dealt with in detail elsewhere (Hengstenberg, Penberthy, and Morse, Federation Proc., *in press*); it has been possible to show complementation between extracts of $z^- cnc^+$ and $z^+ cnc^-$ mutants. To further illustrate the potentialities of the system, fractionation of extracts into cell membranes and soluble constituents shows that the defect of the cnc^- mutants is in a membrane component and that the factor missing in the car^- mutants is soluble, separable from membranes by centrifugation, and has a molecular weight in excess of 100,000. The β-galactosidase, purified 25-fold (ammonium sulfate, Sephadex), has been found to have a molecular weight of about 45,000. Purification of this enzyme required the chemical synthesis of the substrate ONPG-6-phosphate (Hengstenberg and Morse, *Carbohydrate Research*, *in press*).

DISCUSSION

Kundig, Ghosh, and Roseman (10) discovered a phosphotransferase system in *E. coli* that leads to the intracellular accumulation of a wide variety of phosphorylated carbohydrates. A similar system appears to occur in *S. aureus*, and to be the basis of the car^+ phenotype, previously considered to be concerned with a membrane carrier. The two phosphotransferase systems appear to be different, however, in that the system in *S. aureus* acts on lactose, whereas that in *E. coli* has not been shown to do so. The two microorganisms appear to differ further in the enzymes hydrolyzing lactose: that from *E. coli* has a molecular weight of 540,000 (1) and acts only on nonphosphorylated lactose; that of *S. aureus* has a molecular weight of 45,000 and is inactive on the nonphosphorylated β-galactosides. The systems for induction of enzymatic activity also appear quite dissimilar. Despite these differences there are similarities: the genes involved are closely linked and the gene orders may be the same; there is a membrane-bound enzyme involved in each case, the *y* gene product in *E. coli* and the *cnc* gene product in *S. aureus*. It can only be speculated what advantages these similarities have from the evolutionary standpoint and from what common ancestor they may have originated.

The clarification of lactose metabolism provided here also provides clarification of some observations made on the *car* system. In a previous study (4), genetic evidence for a common, pre-

sumed carrier step was sought among mutants in which the kinetics of ONPG hydrolysis by intact cells was modified. It was hoped that mutants would be found that would give a linear response (increase in rate of hydrolysis) to increasing concentration of ONPG, as was described by Herzenberg (7) for *y* mutants of *E. coli*. None was found, although mutants with altered maximal velocities were isolated, described, and shown genetically to be mutant for a function distinct from either the *car* gene product or the β-galactosidase. The failure to find mutants giving a linear response can be understood now that it is known that the staphylococcal β-galactosidase cannot act upon free ONPG but only on the phosphorylated derivative, presumably formed by the *cnc* gene. It is doubtful that this gene's product could be altered easily so as to give a linear response. This supposition, however, creates another problem, that of the linear velocity increase shown (4) at high concentrations of ONPG by intact wild-type cells. With the assumption that the phosphotransferase system in *S. aureus* is similar to that of *E. coli*, it is difficult to explain such a response.

The staphylococcal β-galactosidase, a new enzyme, may have taxonomic value and serve to identify staphylococci uniquely. Experiments to test for its presence in other species of bacteria are in progress.

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