GENETIC REGULATION OF MALTOSACCHARIDE UTILIZATION IN PNEUMOCOCCUS[1]

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BACTERIA respond in versatile fashion to the nutritional resources of their environment by regulating the function of their genes. Examination of the mechanisms of such regulation, mostly in Enterobacteria, has revealed the existence of specific regulatory genes and of several different patterns of control (reviewed by MAAS and MCFALL 1964). Similar regulatory mechanisms may be responsible for other aspects of cellular organization and for the development of multicellular organisms. It seems justifiable, therefore, to extend the analysis of regulation to new systems, particularly in forms other than Enterobacteria.

The system for maltosaccharide utilization in pneumococcus (*Diplococcus pneumoniae*) consists of at least five components. Separate permeases transport each of the sugars—maltose, maltotriose, and maltotetraose—into the cell. Two enzymes, amylomaltase and phosphorylase, participate in the metabolism of these sugars within the cell. All five cellular components are coordinately controlled by a regulatory gene. Genetic analysis, accomplished by DNA-mediated transformation, served to locate the positions of the various genes and to elucidate the mechanism of regulation.

MATERIALS AND METHODS

Chemicals: Commercially obtained maltose was recrystallized twice from ethanol to reduce the proportion of glucose from 0.5% to 0.02%. Maltotriose, maltotetraose, and maltopentaose, formed by the action of pneumococcal amylomaltase on maltose, were separated on a charcoal column (PUTMAN 1957). Sugar concentrations were determined with the anthrone reagent (ASHWELL 1957). Radioactive maltosaccharides were prepared by the action of amylomaltase on maltose-U-C^{14}, 4.45 mc/mmole; they were separated by paper chromatography (LACKS and HOTCHKISS 1960a). All other chemicals were commercial products. To destroy contaminating maltase activity, solutions of bovine serum albumin (Armour) were brought to pH 3.5, heated 15 minutes at 100°C, and then neutralized. Insoluble material in Merck reagent dextrin was removed by centrifugation. Tris stands for *tris*-(hydroxymethyl) aminomethane.

Media: The basal medium contained casein hydrolysate, amino acids, vitamins, salts, albumin, and catalase (LACKS 1966). A low-phosphate medium was similarly composed (LACKS 1962). Cultures were grown for conservation and transformation in the basal medium supplemented with fresh yeast extract and glucose. Sugar concentrations were 0.2%, unless otherwise indicated. For selective growth yeast extract was omitted and the appropriate sugars or drugs were added. The *sul-d* marker was selected with sulfanilamide at 0.1 mg/ml. Selective media containing more than one sugar were composed as follows. Glucose and maltose: 0.3% and 0.1%, respectively. Low glucose, high maltose: 0.04% and 0.2%. Low sucrose, high maltose: 0.02% and 0.2%. Low

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TABLE 1

Revised nomenclature of mutations in the amylomaltase gene

Revised	Original	Revised	Original	Revised	Original
malM503	Mc	*malM533*	V13	*malM564*	E4
MP504	Md	*MP534*	V14	*M565*	E5
MP505	Me	*M535*	V15	*M566*	E6
M506	Mf	*M536*	V16	*M567*	E7
M507	Mg	*M537*	V17	*M568*	E8
M508	Mh	*M538*	V18	*MP569*	E9
DM509	Mi	*M541*	N1	*M571*	O1
M510	Mj	*M542*	N2	*M572*	O2
M511	Mk	*M543*	N3	*M573*	O3
MP512	Ml	*M544*	N4	*M574*	O4
M513	Mm	*M545*	N5	*M575*	O5
M514	Mn	*M546*	N6	*M576*	O6
M515	Mo	*M547*	N7	*DMP581*	P1
M516	Mp	*M548*	N8	*M583*	P3
M517	Mq	*M549*	N9	*M584*	P4
M518	Mr	*M550*	N10	*M585*	P5
M521	V1	*M551*	N11	*M586*	P6
M522	V2	*M552*	N12	M587	P7
M524	V4	*M553*	N13	*RDMP591*	T1
DM525	V5	*M556*	H2	*M592*	T2
M526	V6	*M557*	H3	*M593*	T3
M527	V7	*M558*	H4	*M594*	T4
MP528	V8	*M561*	E1	*M595*	T5
M530	V10	*M562*	E2	*M596*	T6
M532	V12	*M563*	E3	*M597*	T7

sucrose, high glucose, high maltose: 0.02%, 0.2% and 0.2%. For the isolation and quantitation of colonies of desired phenotype, cultures were plated in medium containing 1% agar. Since pneumococci grow best when shielded from air, platings were overlaid with agar medium.

Bacterial strains and nomenclature: The wild-type strain of pneumococcus, R6, and a derivative bearing the *sul-d* marker for sulfonamide resistance were obtained from DR. R. HOTCHKISS. All mutants were derived from R6. Previously described mutations in the structural gene for amylomaltase (LACKS 1966) have been renamed (Table 1) to conform to the recommendations of DEMEREC, ADELBERG, CLARK and HARTMAN (1966). Since the phenotypic effect of the mutation V11 is not fully understood, its designation remains malV11, but this does not imply that it represents a distinct genetic locus. Otherwise, locus designations reflect results of the present work. Genes for the maltose, maltotriose, and maltotetraose permeases are designated *malB, C,* and *D,* respectively. *malP* refers to the phosphorylase gene, and *malR* to the regulatory gene of the system. Constitutive mutations are designated R^c and noninducible mutations, R^n. The *mal* region is defined as that genetic segment which includes genes *R, D, M,* and *P,* and which is deleted in mutant *591.*

Origin of mutants: Three mutations in the gene for maltose permease, *B201, B202,* and *B204,* arose spontaneously in stocks of *M517, M583,* and *M571,* respectively. On routine passage the double mutant cells outgrew the others, so that when these stocks were transformed with wild-type DNA, they gave rise to amylomaltase-positive transformants that formed tiny rather than large colonies in medium containing crude maltose. Pure clones of the double mutants were isolated from these stocks. Strains containing only the permease-negative mutation were obtained by transforming double mutants with wild-type DNA and selecting in medium containing glucose

and maltose, since cells that are B^-M^+, unlike B^-M^- or B^+M^- cells, can use glucose in the presence of maltose. Mutant *B203* was obtained directly by treating a wild-type culture with 1-methyl-3-nitro-1-nitrosoguanidine and screening individual clones first for inability to use maltose and then for inability to take it up from the medium.

The phosphorylase-negative mutant, *P601*, was isolated by transforming M514 with wild-type DNA treated with nitrous acid and screening the maltose-positive transformants for the presence of phosphorylase. Of 120 clones tested, one was negative.

One noninducible mutant, *R3*, was isolated from an ultraviolet irradiated wild-type culture by the penicillin selection technique (LEDERBERG and DAVIS 1950) on the basis of its inability to grow in maltose medium. The other noninducible mutations were obtained in strain M511 as spontaneous mutants able to use glucose in the presence of maltose. (The inhibition by maltose of glucose utilization in M^- cells apparently requires induction of the system, since R^+M^- or R^cM^- cells are inhibited, but R^nM^- cells are not.) In one experiment, cultures of M511, grown in glucose medium from small inocula to $A_{650} = 0.4$, were diluted 4-fold into medium containing glucose and maltose and incubated 4 hours longer at 37°C. A sample of each culture (0.1 ml) was plated in medium containing glucose and maltose. After 40 hours at 37°C, ~ 20 colonies appeared per plate. One colony was picked from each plate and tested for the presence of a second maltose-negative mutation loosely linked to *M511* and closely linked to *R3*. Ten out of twelve isolates contained such a mutation. To obtain strains bearing only the R^n mutation, wild-type cells were transformed with DNA from the double mutant and plated in low glucose, high maltose medium. In this medium R^+M^- does not grow, R^+M^+ gives large colonies, while both R^nM^- and R^nM^+ give small colonies. The R^nM^+ type was identified by its ability to give maltose-positive recombinants when transformed with R^+M^- DNA.

Constitutive mutants were obtained as maltose-positive revertants of noninducible strains. Such revertants occur spontaneously at a frequency of ~ 10^{-6}; the majority of them show high levels of amylomaltase when grown in the absence of maltose. Ultraviolet irradiation increases the frequency of revertants to ~ 10^{-3}; virtually all of these revertants are constitutive. In practice, a culture of R^n6 was grown to $A_{650} = 0.6$ and irradiated with a General Electric germicidal lamp to 10^{-3} survival. Samples of 0.5 ml were diluted into 8 ml fresh glucose medium and incubated for 5 hrs. From each subculture, 0.1 ml was plated in maltose medium. About 30 colonies appeared per plate. One colony from each plate was picked and tested for constitutive synthesis of amylomaltase. Each isolate was then tested for the presence of the original R^n6 mutation by using its DNA to transform an M^- strain, such as DM525, and plating the transformed cultures in low glucose, high maltose medium. Small colonies, of genotype R^nM^- or R^nM^+, revealed the presence of the R^n6 mutation in the constitutive isolate. The constitutive isolates that arose from single-site mutations still contained R^n6. The mutation R^c19 was isolated free from the accompanying R^n6 by crossing double mutant (R^cR^n) DNA with DM525 cells and testing large colonies from a plating in low glucose, high maltose medium, first for constitutivity and then for the absence of R^n6. Constitutive mutants that originally failed to segregate out R^n6 all harbored deletions, some quite lengthy, of the region containing R^n6. In one experiment, such deletions accounted for 21% of all the constitutives obtained.

Construction of strains: The amylomaltase-negative mutation *M511* was introduced into B^- or R^nR^c strains by transforming them with the M^- DNA and selecting small colonies in medium containing low sucrose, high glucose, and high maltose. The parental cells and R^nR^+ recombinants all form large colonies in this medium. *M511* was similarly transferred into R^c19 and into those constitutive mutants where R^n6 was deleted; here, low sucrose, high maltose medium sufficed for selection. A strain carrying R^c19 and *P601* was formed by transforming *R19*, *M511* cells with *P601* DNA and screening the maltose-positive transformants for the absence of phosphorylase. Although strain V11 is inhibited by maltose from growing in the presence of glucose, the inhibition is not as strong as with M^- strains. Penicillin selection in medium containing glucose and maltose, therefore, allowed the isolation of a V11, *M594* double mutant from a culture of V11 transformed with *M594* DNA. The *sul-d* mutation was introduced by transformation into all DNA donor strains used in recombination analysis so that it could serve as a reference marker.

DNA preparation: Purified DNA, prepared according to HOTCHKISS (1957), was used in the

construction of certain strains and in treatments with nitrous acid. In general, however, crude lysates were used without further purification. Crude lysates were prepared by incubating 4×10^9 cells in 0.2 ml of solution containing 0.1 M sodium citrate, 0.15 M NaCl, and 0.1% sodium deoxycholate for 5 minutes at 30°C. The lysates were then diluted with 2 ml 0.15 M NaCl and stored at —20°C. Although crude lysates did not differ appreciably from purified DNA in transforming activity, all the recombination experiments reported here were carried out with crude preparations.

Transformation procedure: Transformable cultures were grown to $A_{650} \sim 0.1$ and stored frozen after addition of 10% glycerol (Fox and Hotchkiss 1957). Thawed cultures were diluted 50-fold into fresh medium and treated with DNA at ~ 1 μg/ml for 30 min at 30°C. Addition of pancreatic deoxyribonuclease to 1 μg/ml terminated uptake. The cultures were then incubated for 90 min at 37°C before plating in selective media. When negative transformants were sought, as in the construction of strains, cells were allowed to react with DNA for 1 hr at 30°C; they were then incubated for 3 hr at 37°C, to allow segregation of negative colony-forming units, before plating.

Genetic recombination analysis: Crosses between the maltose-negative B^- or R^n mutants were carried out and analysed in the manner previously used for the maltose-negative M^- and V11 mutants (Lacks 1966). A given mutant was transformed with wild-type DNA and with DNA from other mutants. Transformants were selected in maltose medium and in sulfanilamide medium. The ratio of maltose-positive to sulfonamide-resistant transformants with wild-type DNA gives the *integration efficiency* of the donor *mal*+ marker. With mutant DNA, the ratio of maltose-positive recombinants to *sul-d* transformants gives the *recombinant frequency* for the mutant cross. The *recombination index*, that is, the ratio of the recombinant frequency to the integration efficiency, measures the distance between the mutant sites.

Crosses between R^c mutants were analysed in analogous fashion. Recipient cells were always *R^n6, R^cx, M511* in genotype. Such cells cannot grow in medium containing glucose and maltose because the maltose inhibits use of the glucose. Nonconstitutive transformants, however, if they retain the R^n mutation, can grow in this medium. The frequency of such transformants (relative to *sul-d* transformants) with *R^n6, M511* DNA gives the integration efficiency for the R^+ marker corresponding to the constitutive mutation in the recipient. Recombinant frequencies were determined from crosses of the type: *R^n6, R^cy, M511* DNA → *R^n6, R^cx, M511* cells. Failure to give any recombinants able to grow in glucose and maltose indicated overlapping of donor and recipient R^c mutations. Such failure to recombine was used to map the extent of multisite mutations, or deletions, in the regulatory gene, in crosses of the type: *del(R^n6)R^cz, M511* DNA → *R^n6, R^cx, M511* cells. Since some cells in the untransformed population either revert or otherwise escape inhibition by maltose, recombinant frequencies $< 5 \times 10^{-4}$ were not measurable.

Overlapping of constitutive deletions with the sites of noninducible mutations was determined in crosses of *del R^c* DNA → *R^n* cells. Maltose-positive transformants were selected and tested for constitutive formation of amylomaltase. The occurrence of nonconstitutive transformants indicated that the donor deletion does not encompass the site of mutation in the recipient. The overlap of *R* gene deletions with deletions extending into the *M* gene was similarly determined. For example, failure to detect nonconstitutive maltose-positive transformants in the cross *RD36* DNA → *DM509* cells showed that donor and recipient deletions include sites in common. Overlapping of *M* gene deletions with the site of mutation *P601* was demonstrated by the inability of *P601* DNA to transfer the multisite marker to recipients cells harboring the deletion without also transferring the donor mutation. In this test maltose-positive transformants were tested for phosphorylase activity.

Growth with maltosaccharides: Cultures were grown in medium containing either glucose or maltose to $A_{650} \sim 0.2$. The cells were centrifuged at 3°C, washed once with medium, and resuspended in medium to $A_{650} \sim 0.02$. Glucose or maltosaccharides were added to 2-ml portions to give sugar concentrations of 0.5 mg/ml. This amount of sugar allows growth to $A_{650} \sim 0.3$. Absorbance of the cultures was measured before incubation at 37°C and at 20 min intervals, therafter, by transfer to cuvettes maintained at 37°C in a Beckman spectrophotometer. Absorb-

ance is proportional to cell concentration over the range followed. An absorbance at 650 mμ of 0.1 is equivalent to $\sim 8 \times 10^7$ colony-forming units or ~ 20 μg protein per ml.

Maltosaccharide uptake: Accumulation of maltosaccharides was measured in glucose-grown cells suspended in either glucose medium or buffered saline at densities ranging from $A_{650} = 0.2$ to 1.0. The buffered saline, which does not allow growth, contained 0.05 M potassium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, 0.2% glucose, 0.05% albumin, and 3 units catalase per ml, at pH 7.5. Radioactive and unlabeled maltosaccharides were added to give 2×10^3 counts per min and total concentrations from 40 to 300 mμmoles per ml. After incubation, samples were diluted into chilled medium. The cells were centrifuged, washed once by resuspension in fresh medium, suspended finally in 0.05 M NaCl, and plated for counting. In some experiments samples were filtered and washed on Millipore discs, which were then dried and counted directly. Procedural details are specified in the tables. Kinetics of maltosaccharide uptake were followed by mixing, at 0 time, 5 ml of culture with 2 ml of sugar solution, both preincubated at 30°C, and then withdrawing 1-ml samples into 2 ml chilled medium at intervals.

Intracellular contents were analysed by paper chromatography. Lysates for spotting were prepared by incubating washed cells for 5 min at 30°C in 0.05 M NaCl containing 0.1% sodium deoxycholate. After autoradiography of the chromatograms, sections of the paper corresponding to individual maltosaccharides were excised for quantitative determination of radioactivity.

Enzyme assays: For determinations of enzyme activity, 50-ml cultures were harvested at $A_{650} \sim 0.6$. The cells were washed with 10 ml of the assay diluent (3 mM glutathione, 10 mM NaF, 100 mM NaCl, 50 mM Tris·HCl pH 7.5) and resuspended in 0.5 ml of this same solution containing, also, 0.1% DOC. The cells lysed on incubation at 30°C for 10 min. Protein was determined, in samples diluted in water, by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951), with bovine albumin as the standard. Amylomaltase activity was measured by the release of glucose in samples incubated with 2% maltose at 30°C. Glucose was determined with glucose oxidase as previously described (LACKS and HOTCHKISS 1960b). One unit of amylomaltase releases one mμmole of glucose per min.

Kinetics of amylomaltase induction were followed in washed, glucose-grown cells that were suspended at $A_{650} = 0.1$ in medium containing 0.2% glucose, 0.2% maltose, or both, and incubated at 37°C. Samples were chilled at 0, 20, 40, 60, and 80 min. The A_{650} of 2-ml portions was measured, and the cells from 5-ml portions were washed, lysed, and assayed for amylomaltase.

Individual clones were screened for constitutive formation of amylomaltase by growing cultures of colonies picked into sucrose medium to $A_{650} \sim 3$. One drop of a mixture containing 0.2% sodium deoxycholate, 3 mM 2-mercaptoethanol, and 5% maltose was added to 0.2 ml of culture. After 120 min at 30°C, glucose was determined as before. Constitutive clones gave dark color ($A_{420} > 1.0$); nonconstitutives gave light color ($A_{420} < 0.2$).

Phosphorylase was measured by the release at 37°C of inorganic phosphate from glucose-1-phosphate in the presence of dextrin. Assay mixtures consisted of samples of a cell lysate, 3 mM glutathione, 2 mM NaF, 20 mM NaCl, 1.6 mM glucose-1-phosphate, 2% soluble dextrin, and 50 mM Tris·HCl, pH 7.5, in 1 ml. The reaction was terminated by addition of 1 ml of 0.1 M ammonium acetate, pH 4.3. Color was developed, according to the method of LOWRY and LOPEZ (LELOIR and CARDINI 1957), by successive addition of 0.2 ml 1% ascorbic acid and 0.2 ml of 1% molybdic acid and incubation for 60 min at 37°C. Absorbance was measured at 700 mμ. In some experiments phosphorylase activity was measured in the presence of 0.7% soluble starch (Calbiochem) instead of dextrin; the rate of phosphate release with 0.7% starch was one-sixth of that obtained with 2% dextrin. One unit of phosphorylase corresponds to the release of 1 mμmole of inorganic phosphate per min with dextrin as the glucosyl acceptor.

Screening for phosphorylase was accomplished by picking colonies into 2 ml low-phosphate medium containing 0.2% maltose. When these cultures reached maximal turbidity, they were centrifuged and the cells were suspended in 1 ml of a mixture composed of 0.1% sodium deoxycholate, 0.1 M NaCl, 1 mM glucose-1-phosphate, 0.9% soluble starch, and 0.05 M Tris·HCl, pH 7.5. After incubation for 3 hrs at 37°C, the reaction was terminated and the color due to inorganic phosphate was revealed. Phosphorylase-positive clones gave dark color ($A_{700} > 1.0$); phosphorylase-negative clones gave light color ($A_{700} < 0.2$).

RESULTS

Growth with maltosaccharides: Wild-type pneumococci can grow with maltosaccharides—maltose, maltotriose, or maltotetraose—as the sole source of carbohydrate (Figure 1). When cells grown on glucose, and hence not induced for the maltosaccharide utilization system, are transferred to maltosaccharide medium, they grow, initially, as fast as in glucose medium. After one doubling, however, growth lags for ~ 40 min., after which it resumes at a rate equal to or slightly greater than that with glucose (Figure 1a). Cells induced for the system, either by prior growth in maltose or by the presence of a constitutive mutation in the regulatory gene, grow rapidly without lag (Figure 1b, c).

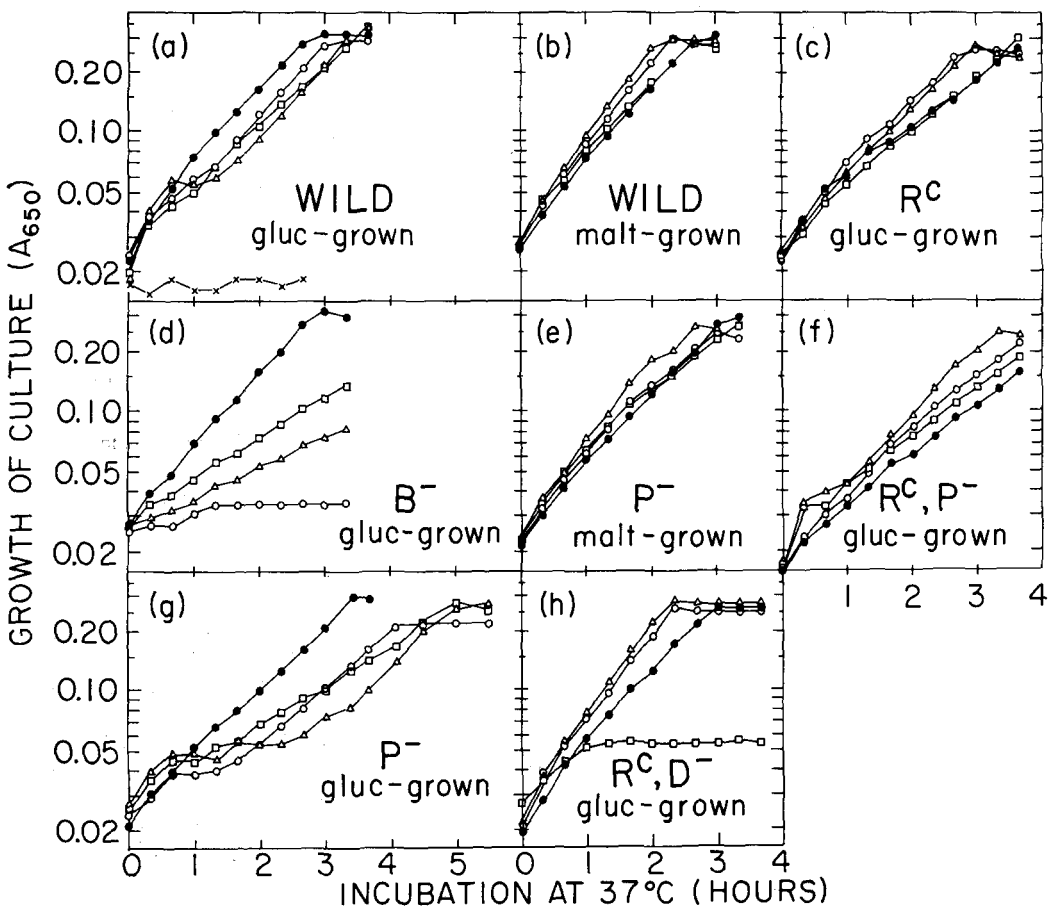

FIGURE 1.—Growth of pneumococcal strains with glucose and maltosaccharides. (a) wild type; glucose-grown. (b) wild type; maltose-grown. (c) *R19*, constitutive; glucose-grown. (d) *B201*, maltose permease-negative; glucose grown. (e) *P601*, phosphorylase-negative; maltose-grown. (f) *R19*, *P601*, constitutive, phosphorylase-negative; glucose-grown. (g) *P601*, phosphorylase-negative; glucose-grown. (h) *R34*, constitutive, maltotetraose permease-negative; glucose-grown. Growth with glucose (●), maltose (○), maltotriose (△), maltotetraose (□), no sugar (x).

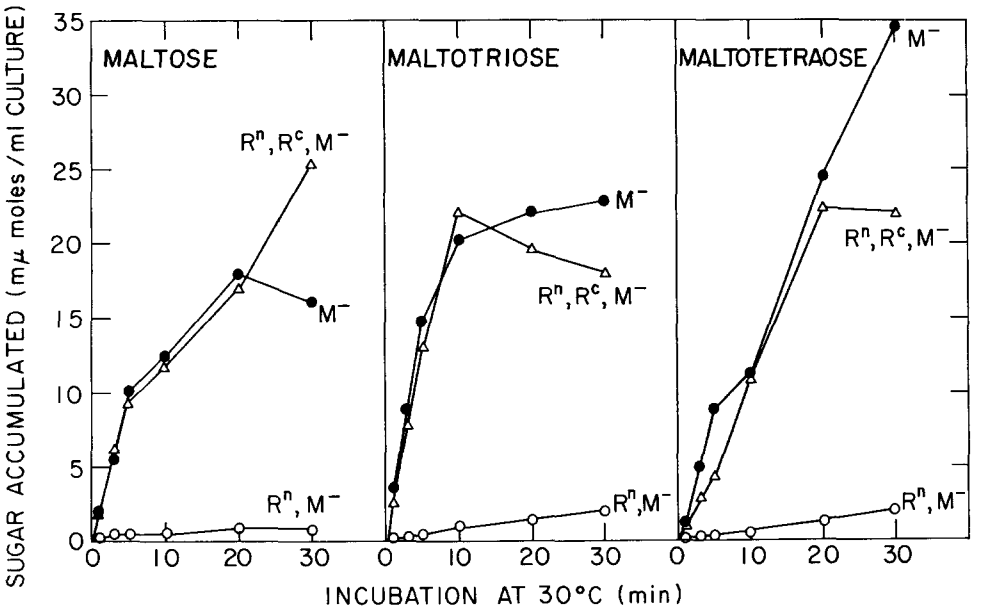

FIGURE 2.—Kinetics of maltosaccharide uptake. Glucose-grown cells suspended in glucose medium at 2.4 × 10^8 colony-forming units per ml ($A_{650} = 0.3$) were incubated with C^{14}-labeled maltosaccharides at 100 mμmoles per ml. Strains: *M511* (●); *R6, M511* (○); *R6, R19, M511* (△).

Maltosaccharide transport: The ability of cells to take up maltosaccharides from the medium can be measured by the accumulation of radioactive sugars in strains lacking amylomaltase, the enzyme essential for the ultimate metabolism of maltosaccharides. Cells lacking this enzyme concentrate maltosaccharides to levels a thousand-fold greater than the external concentration. The uptake requires a source of energy, such as glucose, in the medium. Maltose and maltotriose are taken up by cells suspended in buffered saline as well as by cells in growth medium, but maltotetraose is only taken up well under the latter conditions. The kinetics of uptake in glucose medium at 30° C (where the cell-doubling time is > 80 minutes) are shown in Figure 2. Subsequent data indicate that separate genes, and therefore separate permeases, are responsible for the transport of maltose, maltotriose, and maltotetraose, respectively.

Table 2 shows the uptake of maltose in various *M* mutants. Addition of a mutation at the *B* locus prevents accumulation of this sugar. Lengthy deletions in the vicinity of the *M* gene, such as occur in mutants *569* and *591* (LACKS 1966), do not interfere with maltose uptake. One of these deletions, *569*, probably extends far to the right of *M* since it begins within that gene. The other deletion, *591*, extends both to the left and the right, and it also encompasses the gene for maltotetraose permease. Chromatographic analysis of cellular content showed that, in all cases, the maltose taken up persisted unchanged.

Strains that carry only the *B* mutation also fail to take up appreciable amounts

TABLE 2

Maltose uptake by mutant strains

Genetic constitution	Maltose accumulated (mμmoles/ml)
M517	48
B201, M517	<2
M511	172
B202, M511	<2
B203, M511	<2
M571	126
B204, M571	<2
MP569	145
RDMP591	111

Glucose-grown cells were suspended in buffered saline at 8×10^8 colony-forming units per ml. Samples were incubated 30 min at 37° with C^{14}-labeled maltose at 286 mμmoles per ml.

of maltose from the medium, and they do not grow in the presence of maltose (Figure 1d). They grow with maltotriose and maltotetraose, but at subnormal rates. It is not clear why this is so, for *B* mutants accumulate these sugars (Tables 4 and 5), and the maltose permease, as shown later, is not required for induction. A conceivable explanation is that maltose, formed as an intermediate in the metabolism of the higher oligosaccharides, leaks out of the cell and fails to reenter.

Genetic analysis of the four maltose permease-negative mutations showed that they occur at distinct, but linked, sites (Table 3). The mutants recombined to give maltose-positive transformants, but at lower frequencies than obtained by transforming mutants with wild-type DNA. With the *mal* region deletion, *591*, as donor, frequencies were as high as with wild type, which corroborates the phenotypic evidence that the *B* gene is not located within the region corresponding to *591*, and shows, further, that its locus cannot even be closely linked to the *mal* region. The *B* locus is mapped in Figure 3. Distances, determined as recom-

TABLE 3

Recombination between mutations in the maltose permease gene

DNA donor strain	Frequency of maltose-positive transformants* with recipient strains			
	B201	*B202*	*B203*	*B204*
Wild type	0.575	0.089	0.070	0.047
RDMP591	0.579	0.133	0.070	0.081
B201	<0.00005†	0.031	0.0117	0.0023
B202	0.171	<0.001	0.0169	0.0186
B203	0.0655	0.013	<0.00005	0.0092
B204	0.0147	0.046	0.0092	<0.00002

* Ratio of maltose-positive to sulfonamide-resistant transformants.

† Maximal values represent ratios of spontaneous revertants in recipient cultures not treated with DNA to sulfonamide-resistant transformants in parallel, DNA-treated cultures.

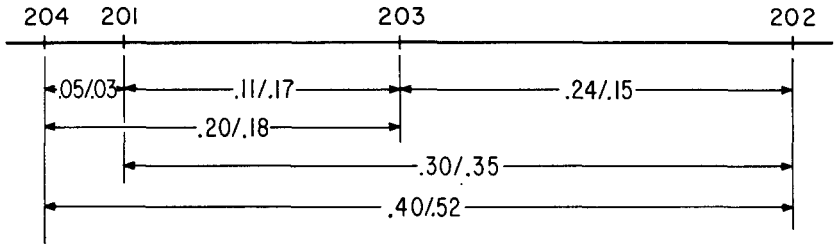

FIGURE 3.—Map of the maltose permease gene. The heavy line represents the genetic structure; vertical marks indicate the sites of *B* mutations. Distances determined as recombination indices are shown for reciprocal crosses between mutant pairs. The value nearest the arrowhead corresponds to the cross where the recipient cell contains the mutation indicated at that arrowhead.

bination indices, agree fairly well in reciprocal crosses, and they are essentially additive over the extent of the locus.

Maltotriose uptake by various mutant strains is indicated in Table 4. Here, too, the accumulated sugar remains unchanged. The ability to accumulate maltotriose is present in cells lacking the maltose and maltotetraose permeases, as well as in cells in which the entire *mal* region is deleted. The gene for maltotriose permease must, therefore, be located elsewhere.

The location of the maltotetraose permease gene, *D*, was determined from the accumulation data in Table 5; its position is indicated, later, in Figure 7. Maltotetraose taken up by phosphorylase-negative cells persists unchanged; however, in phosphorylase-positive cells, sampled after 30 min at 37°, about half of the radioactivity taken up is converted to maltotriose. Deletions that extend into the central portion of the *mal*-region, either from the right end of the *R* gene or from the left end of the *M* gene, prevent maltotetraose accumulation. Figure 1h shows that a *D* mutant grows normally with maltose and maltotriose, but hardly at all with maltotetraose. (The latter growth represents only 10% of that attainable with the amount of sugar added, and it is probably due to a contamination of that order by maltotriose in the maltotetraose preparation, since the two sugars are incompletely resolved on the charcoal column used to separate the oligo-

TABLE 4

Maltotriose uptake by mutant strains

Genetic constitution	Maltotriose accumulated (mμmoles/ml)
M517	98
B201, M517	135
DM525	108
MP504	120
RDMP591	117

Glucose-grown cells were suspended in glucose medium at 8×10^8 colony-forming units per ml. Samples were incubated 30 min at 37°C with C^{14}-labeled maltotriose at 155 mμmoles per ml.

TABLE 5

Maltotetraose uptake by mutant strains

Genetic constitution		Maltotetraose accumulated (mμmoles/ml)	Genetic constitution		Maltotetraose accumulated (mμmoles/ml)
Experiment I (Maltotetraose at 43 mμmoles/ml)			Experiment II (Maltotetraose at 160 mμmoles/ml)		
M511 coupled with *R*-gene deletions:			*M*-gene deletions:		
left-end	*23*	21	through, past *R* gene	*591*	<1
	26	23	past *V11*	*581*	<1
	28	24	left-end, past *V11*	*509*	<1
right-end	*34*	<1		*525*	<1
	35	<1	through, not past *V11*	*505*	82
	38	<1		*534*	82
through	*25*	<1	right-end	*512*	95
	29	<1		*569*	88
	31	<1	internal	*503*	93
	33	20		*515*	70
	36	<1	*M*-gene single-site mutations:		
	37	<1		*511*	80
	39	<1		*594*	76
			Maltose-permease negative:		
				B201, M517	128
			V11-containing strain:		
				V11, M594	98

Glucose-grown cells were suspended in glucose medium at 8×10^8 colony-forming units per ml. Samples were incubated 30 min at 37° with C^{14}-labeled maltotetraose.

saccharides.) Since D^+ but not D^- strains can grow with maltopentaose, also, the same permease appears to transport both the tetra- and pentasaccharides.

Maltosaccharide metabolism: An intracellular enzyme, amylomaltase, is essential for the utilization of maltosaccharides. Amylomaltase activity is lacking or severely reduced in mutants at the *M* locus, which appears to be the structural gene for the enzyme. Strains carrying such mutations are unable to grow with any of the maltosaccharides. The absence of amylomaltase also confers several secondary phenotypic characteristics. One is the inhibition by maltose of growth in the presence of glucose. On addititon of maltose to a culture growing in glucose medium, the rate of growth declines steadily, with some inhibition already apparent by 30 min. Amylomaltase-negative cells fail to form colonies in medium containing glucose and maltose, but they form normal colonies with glucose alone or in sucrose medium with or without maltose present. Another secondary characteristic of *M* gene mutants is "self-induction," that is, the induction of other components of the maltosaccharide system when cells are grown with glucose, which is not normally an inducer. The *M* gene has been mapped previously (LACKS 1966); positions of mutations used in the present work are shown in Figure 7.

The amylomaltase enzyme was purified by successive fractionation of cell extracts on columns of Sephadex G-200 and diethylaminoethyl cellulose until the

peak of protein corresponded with the peak of enzyme activity. From the specific activity of such preparations, 10^5 units per mg protein, calculation shows that the induced level of amylomaltase represents about 1% of the total protein. The purified enzyme can only transfer glucosyl residues from one maltosaccharide to another (or to glucose); it cannot hydrolyse the sugars. The products of its action on maltose, consequently, consist of glucose and a mixture of oligosaccharides.

Pneumococci contain another enzyme, phosphorylase, which, in the presence of phosphate, can attack higher maltosaccharides to produce glucose-1-phosphate and the next lower oligosaccharide homologue. Catalytic activity of the enzyme has been measured in both directions: by the release of inorganic phosphate in the transfer of glucosyl residues from glucose-1-phosphate to the maltosaccharides present in dextrin or soluble starch, and by the formation of glucose-1-phosphate in the reverse, phosphorolytic, reaction. The phosphorylase protein is separable from the amylomaltase on either Sephadex G-200 or diethylaminoethyl cellulose columns; it appears to be smaller and less negatively charged than the amylomaltase protein.

The first indication that the phosphorylase gene is closely linked to the amylomaltase gene was the absence of phosphorylase activity in mutants with deletions extending out of the *M* gene (Table 6). Phosphorylase was absent, however, when deletions extended out of *either* side of the *M* gene. When the single-site, amylomaltase-positive, phosphorylase-negative mutant, *P601*, was obtained, its site of mutation was determined by crosses with various *M* gene deletion mutants as recipients. With *P601* as the donor, if the recipient deletion covers the site of *P601*, all amylomaltase-positive transformants must be phosphorylase-negative. Results of this test are given in Table 6. They locate *P601* to the right of gene *M*, as shown in Figure 7. Since only one single-site, phosphorylase-negative mutation has been mapped, however, the results do not exclude the possibility of another structural gene for phosphorylase located to the left of gene *M*. With M^-

TABLE 6

Location of phosphorylase gene from transformation of M-*gene mutants by* P601 *DNA*

Description of recipient strain			$M+\ P+$ recombinants	
M-gene mutation	Location of mutation in *M*-gene	Phosphorylase content*	total $M+$ transformants	Recombination index†
511	left-end single-site	390	0.84	0.61
594	right-end single site	610	0.59	0.19
538	right-end deletion	540	0.55	0.18
509	left-end deletion	<5	0.50	0.21
525	left-end deletion	<5	0.45	0.08
591, 581, 534 or *505*	through deletions	<5	<0.05	
504, 512, 528 or 569	right-end delections	<5	<0.05	

* Units per mg protein for cells grown in the presence of sucrose and maltose.

† Calculated as the ratio of $\left(\frac{M+\ P+}{\text{total } M+} \times \frac{\text{total } M+}{sul\text{-}d}\right)$ to the integration efficiency (with wild-type DNA) of the corresponding $M+$ marker.

mutations that do not overlap with *P601*, the ratio of M^+P^+ recombinants to total M^+ transformants does not directly reflect distances between sites because the low efficiency, nitrous acid induced P^- marker excludes M^+ integration (LACKS 1966). Distances can be calculated as recombination indices, however, and the values obtained are listed in Table 6.

The presence of phosphorylase activity is not essential for growth with maltosaccharides. Although uninduced P^- cells show a longer growth lag than the wild type when transferred to maltosaccharide media (Figure 1g), the growth rates of induced or constitutive cultures of strains containing *P601* are the same as those for the corresponding P^+ strains (Figure 1e, f). Since oligosaccharides do not accumulate within P^- cells grown on maltose, pneumococci must possess an alternative pathway for metabolising the glucosyl residues of oligosaccharides.

Coordinate induction: Growth of wild-type pneumococci in maltose increases the level of both amylomaltase and phosphorylase by 20 to 40-fold over the levels present in glucose- or sucrose-grown cells (Table 7). Induction begins immediately on addition of maltose to a culture, and the increase in enzyme activity is

TABLE 7

Amylomaltase and phosphorylase levels in various strains grown in the presence of different sugars

Strain	Sugars present*	Specific activity†	
		Amylomaltase	Phosphorylase
Wild	G	40	18
	S	65	46
	S+M	439	220
	G+M	1180	540
	M	1510	670
R^n6	G	28	14
	S	42	30
	S+M	43	31
	G+M	36	17
R^c19	G	2000	840
	S	1040	580
	S+M	1190	540
	M	1600	730
R^n6,R^c19	G	1510	780
	S	1050	580
	S+M	1100	560
V11	G	18	8
	S	39	12
	S+M	162	62
R^c19,V11	S	138	58
P601	G	56	<6
	S	47	<2
	M	1490	<2

* Symbols for sugars: G, glucose; S, sucrose; M, maltose.
† Units per mg protein.

proportional to the increase in cell mass from the time of maltose addition (Figure 4a). Mutants that are devoid of amylomaltase activity appear to be self-induced when grown on glucose. They contain high levels of phosphorylase (Table 8) and the three permeases (Figure 2). This is not true for sucrose-grown cells, nor for mutants, such as *M541* and *M595*, that contain appreciable residual amylomaltase activity (Table 8). Pneumococci may be able to convert glucose to maltose, enough of which can accumulate in the absence of amylomaltase to induce the system for maltosaccharide utilization. That low internal concentrations of maltose are sufficient for induction is indicated by the rapid and full induction of cells lacking maltose permease (Figure 4b).

Mutations in the regulatory gene affect all five components of the system in coordinate fashion (Table 7, Figure 2). Noninducible mutants, whether grown in maltose or glucose, contain only uninduced levels of enzymes. When amylomaltase-negative derivatives of R^n strains are grown on glucose, there is no self-induction of either phosphorylase or the permeases. Constitutive mutants, on the

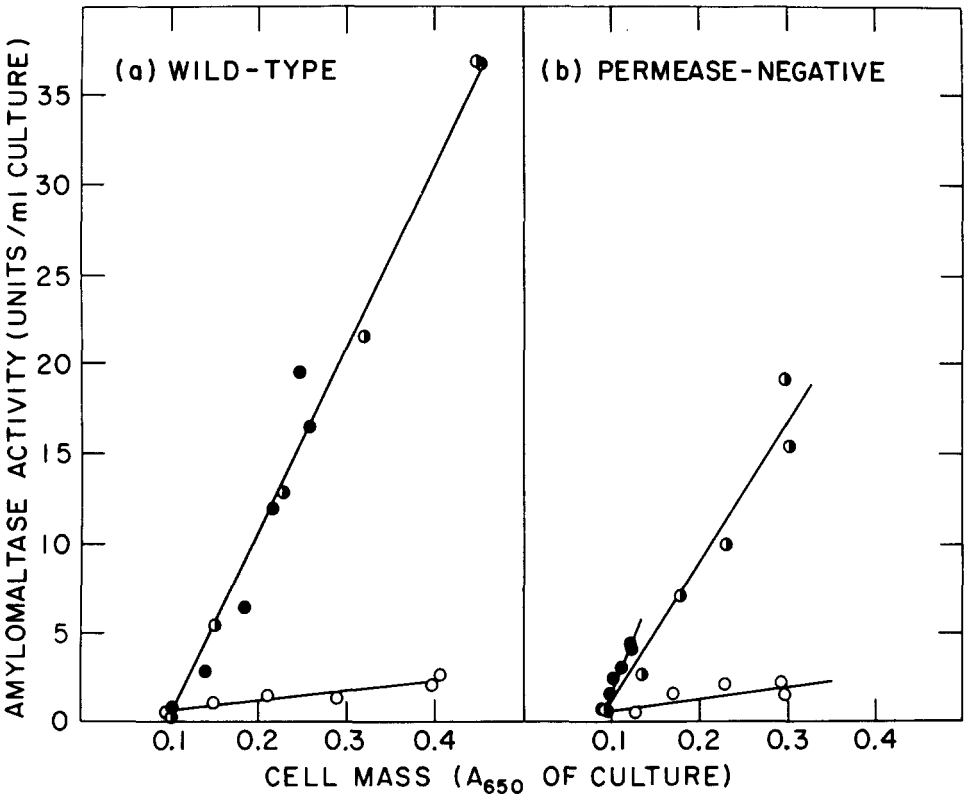

FIGURE 4.—Kinetics of amylomaltase induction. (a) Wild-type strain. (b) Maltose permease-negative strain, *B201*. Washed, glucose-grown cells were incubated at 37°C in medium containing glucose (open circles), maltose (filled circles), and both glucose and maltose (half-filled circles). Samples were assayed at 0, 20, 40, 60, and 80 minutes (duplicate samples were taken at 80 minutes).

TABLE 8

Amylomaltase and phosphorylase levels in M *mutants*

Strain	Sugars present	Specific activity	
		Amylomaltase	Phosphorylase
M547	S	<0.05	20
	G	<0.05	460
	S+M	<0.05	550
M511	S	....	11
	G	....	290
	S+M	....	390
R^n6,M511	S	....	14
	G	....	12
	S+M	....	18
R^c19,M511	S	<0.05	300
M541	S	2.6	24
	G	2.8	30
	S+M	47	540
M595	S	0.7	26
	G	2.9	76
	S+M	23	580

Details as in Table 7.

other hand, contain high levels of all components, irrespective of the sugar on which they are grown. The activity of each component in a constitutive strain is 20 to 40-fold greater than its uninduced level in the wild type or its fixed level in a noninducible strain.

Regulatory gene: Noninducible mutants are unable to grow with maltosaccharides. After transfer to maltose medium, the turbidity of noninducible cultures does not increase more than 30%. Another phenotypic effect of R^n mutations is the reversal of maltose inhibition of glucose utilization in cells that lack amylomaltase.

The close linkage between seven independently isolated noninducible mutations is shown in Table 9, which gives the integration efficiency of the wild-type marker, and the recombination index with mutant DNA, in the transformation of each of the mutants. An appreciable frequency of spontaneous reversion, mainly to constitutive types, sets a lower limit to the detection of maltose-positive recombinants. Mutations *3* and *13* may be identical; but *6* and *8*, which occur at the same site, must be different since they give different integration efficiencies of the wild-type marker. Mutations *5* and *9* are also different; they probably affect separate but very closely linked sites, although in one direction of the cross between them, no recombinants were detectable. The map of noninducible mutations in Figure 5 depicts the mean length of intervals computed from the data in Table 9.

Loose linkage between non-inducible mutations of the regulatory gene and mutations of the amylomaltase gene is manifested in the frequency of cotransfer of wild-type markers in double mutant recipients. For example, $R6^+$ was also

TABLE 9

Recombination between noninducible mutations of the regulatory gene

Recipient R^n strain	Integration efficiency of wild-type marker*	Recombination index with R^n mutant DNA†						
		3	*13*	*7*	*6*	*8*	*5*	*9*
3	0.061	<0.01‡	<0.02	0.026	0.086	0.071	0.107	0.108
13	0.077	<0.007	<0.007	0.038	0.094	0.068	0.137	0.121
7	0.068	0.050	0.037	<0.01	0.072	0.079	0.165	0.143
6	0.685	0.076	0.061	0.039	<0.001	<0.001	0.052	0.073
8	0.244	0.073	0.065	0.043	<0.002	<0.002	0.079	0.078
5	0.485	0.140	0.110	0.097	0.087	0.061	<0.002	0.007
9	0.108	0.133	0.116	0.105	0.086	0.044	<0.003	<0.003

* Ratio of maltose-positive to sulfonamide-resistant transformants with wild-type DNA containing the *sul-d* reference marker.

† Mutant DNA contained the *sul-d* marker. Recombination index is the ratio of maltose-positive recombinants to sulfonamide-resistant transformants divided by the integration efficiency for the wild-type donor marker.

‡ All values are corrected for spontaneous reversion (frequency $\sim 10^{-6}$). Maximum values represent "recombination indices" calculated for twice the background number.

transferred in $\sim$ 40% of the cells transformed to $DM525^+$; $R6^+$ and $M511^+$ were transferred together at a frequency $\sim$ 15% of either marker alone. Both of these frequencies are considerably higher than the expectation for simultaneous independent transformations.

Constitutive mutations in the regulatory locus give high levels of the enzymes and permeases, both when present by themselves and when coupled with noninducible mutations (Table 7; Figure 2). Furthermore, constitutive mutations antagonize the effect of noninducible mutations on maltose inhibition, so that $R^nR^cM^-$ strains are unable to use glucose in the presence of maltose. This property allows selection of R^nM^- recombinants in a cross of the type $R^nR^cxM^- \rightarrow R^nR^cyM^-$ and, hence, genetic analysis of constitutive mutations. The map of single-site constitutive mutations, obtained in this way, is shown in Figure 6. Values, in parentheses, above mutation numbers indicate the integration efficiency of the corresponding wild-type marker. Distances are given, in terms of recombination

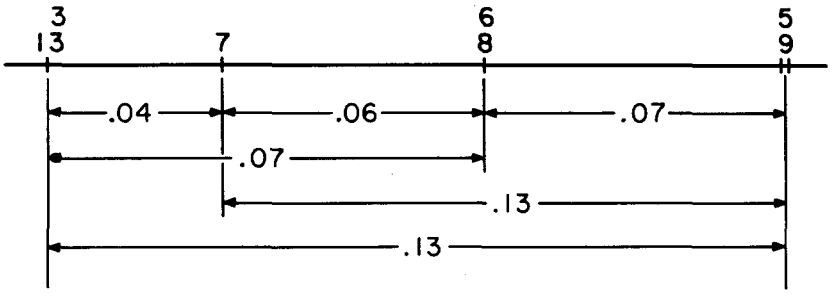

FIGURE 5.—Map of non-inducible mutations of the regulatory gene. The heavy line represents the genetic structure. Vertical marks indicate sites of R^n mutations designated above the line. Lengths of intervals are mean values of recombination indices taken from Table 8.

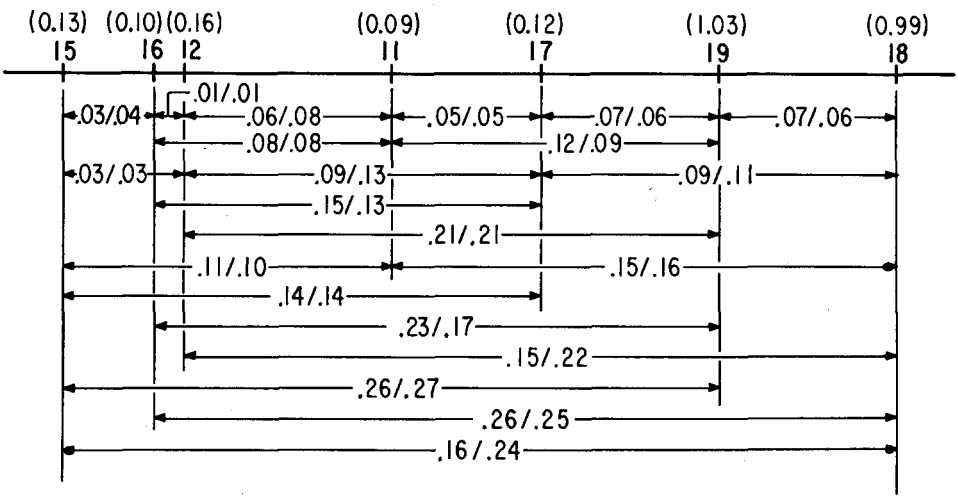

FIGURE 6.—Map of constitutive, single-site mutations of the regulatory gene. The heavy line represents the genetic structure; vertical marks indicate the sites of R^c mutations. In parenthesis, above the numerical designation of each mutation, is the integration efficiency of the corresponding wild-type marker. Distances determined as recombination indices are shown for reciprocal crosses between mutant pairs. The value nearest the arrowhead corresponds to the cross where the recipient cell contains the mutation indicated at that arrowhead.

index, for crosses in both directions. Reciprocal crosses show good agreement, and the distances are additive. Only the relative positions of *12* and *16* are uncertain. The order of sites so deduced was later confirmed by crosses with partial deletions of the locus. The pattern of overlapping that was obtained is shown in Figure 7.

Non-inducible and constitutive mutations occur at sites interspersed in the *R* locus. All the constitutive deletions in which R^n6 was deleted overlap near the middle of the array of single-site constitutive mutations. This, then, must be the location of R^n6. Overlapping of the constitutive deletions with other sites of non-inducible mutations is shown in Table 10. These results indicate that R^n5 lies in a different segment of the *R* locus than do R^n3 and R^n7 (see Figure 7).

The orientation of the array of sites in the *R* locus with respect to genes *D*, *M*, and *P*, is deducible either from the extension of deletions of the right end of gene

TABLE 10

Location of R^n *mutations from recombination behavior with* R^c *deletions*

Recipient strain	Ratio of inducible recombinants (R^+) to total maltose-positive transformants (R^+, R^c and R^nR^c) with DNA from R^c strains				
	23	*26*	*28*	*34*	*38*
R^n3	0	0	0	0	0.08
R^n7	—	0	—	0	0.11
R^n5	0.42	0.32	0	0	—

Number of transformants tested ranged from 24–100. Dash indicates that cross was not tested.

TABLE 11

Overlap of R-*gene deletions with* M-*gene deletions*

Recipient strain containing *M*-gene deletion	Ratio of inducible recombinants (R^+M^+) to total maltose-positive transformants (R^+M^+ and R^cM^+) with DNA from R^c strains									
	33	*37*	*35*	*34*	*25*	*31*	*29*	*38*	*36*	*39*
525	0	0	0	0	0	0	0	0	0	0
581	0.4	0.7	0.1	0.1	0	0	0	0	0	0
509	0.9	0.7	0.9	0.5	0.6	0.6	0.3	0.1	0	0

Ten colonies from each cross were tested for constitutive synthesis of amylomaltase.

R into gene *D* or from the overlap of these deletions with deletions of the left end of gene *M*. Extension into gene *D* was determined by the inability of a constitutive mutant to accumulate maltotetraose (Table 5); overlap with *M*-gene deletions was determined by its inability to give inducible, maltose-positive recombinants in a genetic cross (Table 11). Genes *R*, *D*, *M*, and *P* map, therefore, on a single segment of DNA, the *mal* region (Figure 7). Since *591*, a deletion spanning the whole segment, can be transformed by a DNA "molecule," the size of the segment cannot be exceedingly large—it is probably $< 10^5$ nucleotides long. In Figure 7 only genes *R* and *M* are drawn to scale. The length of intergenic regions on either side of *D* is probably considerably greater than shown. Genes *R* and *D* are not contiguous since *33* and *525* overlap without either of them altering more than one of these genes. Similarly, the phenotypic properties of *V11* indicate that genes *D* and *M* do not abut.

The mal-MP *operon:* The linked pair of genes *M* and *P* appear to form an operon subject to control by the genetic region just to the left of gene *M*. Although

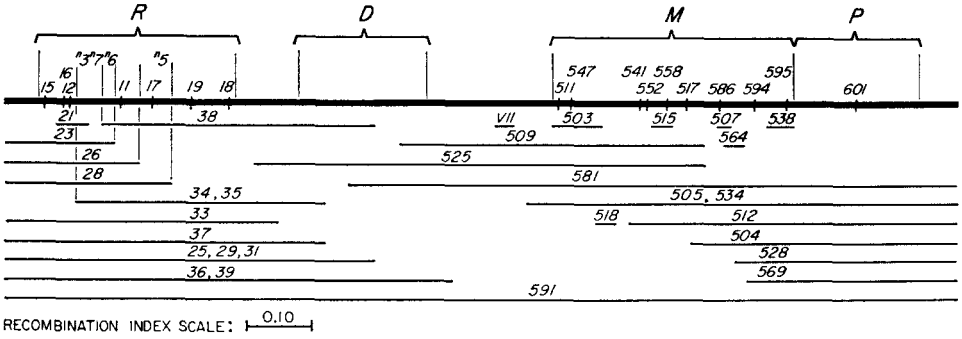

FIGURE 7.—Map of the "*mal* region" containing four genes of the system for maltosaccharide utilization. Genes: *R*, regulatory; *D*, maltotetraose permease; *M*; amylomaltase; *P*, phosphorylase. Positions of single-site mutations are indicated by vertical marks on the genetic structure represented by the heavy line. Numbers above the line refer to these mutations. Only genes *R* and *M*, which have been accurately mapped, are drawn to scale (indicated at the bottom of the figure). The pattern of overlapping of deletions is shown below the genetic structure. In the *R* gene, vertical marks on the genetic structure represent R^c mutations; the approximate location of R^n mutations, in segments defined by deletions, is also indicated.

TABLE 12

Phosphorylase activity in M *mutants*

	Strain	Specific activity (units/mg protein)
Grown in sucrose:	*M552*	21
	M586	23
	M503	16
	M507	20
Grown in sucrose + maltose:	*M552*	650
	M586	310
	M503	330
	M507	210
	M515	730
	M538	540
	M564	710
	M518	430
	M558	530
	M594	610

the phosphorylase gene lies to the right of *M*, deletions that extend only to the left of *M*, such as *509* and *525*, prevent formation of phosphorylase (Table 6). Furthermore, the mutation *V11*, which is probably a small deletion lying to the left of the amylomaltase gene (GHEI and LACKS 1967) reduces the level of function of both the *M* and *P* genes. Such reduction is slight for non-induced synthesis, but the induced or constitutive synthesis of the enzymes is coordinately reduced to about 10% of the constitutive level in the absence of the *V11* mutation (Table 7). That the *M* and *P* genes are more tightly organized together than they are with the rest of the system is indicated by the normal activity of the permease genes in the presence of the mutations just cited (Tables 4 and 5).

Polar effects of mutations in gene *M* on the function of gene *P* were sought by measuring phosphorylase activity in *M* mutants. Measurements were made on non-induced cells grown with sucrose, on induced cells grown with sucrose and maltose, on self-induced cells grown with glucose, and, in one case, on a double mutant carrying a constitutive mutation. Fifty different *M* mutants, grown under one or several of these conditions, were tested. Almost all gave specific activities within 50% of the expected value. Representative values are listed in Tables 8 and 12. Since repeat measurements sometimes varied by as much as 50%, the apparent reduction of phosphorylase activity in certain mutants may not be significant. It appears safe to conclude, however, that mutations in gene *M* exert no *strong* polar effects on the activity of gene *P*.

DISCUSSION

Maltosaccharide utilization in *Escherichia coli* has also been intensively studied. This system is similar to that of pneumococcus in that it includes an amylomaltase (MONOD and TORRIANI 1948), a phosphorylase (DOUDOROFF, HASSID, PUTMAN, POTTER and LEDERBERG 1949), and a permease (WIESMEYER

and COHN 1960), which are all induced by maltose; and the genes for the enzymes are closely linked, while the one for maltose permease maps separately (SCHWARTZ 1966). In *E. coli*, however, a single permease transports not only maltose but also maltosaccharides containing as many as ten glucose residues (SCHWARTZ 1965). Furthermore, the phosphorylase but not the amylomaltase is essential for the metabolism of these oligosaccharides (SCHWARTZ 1965). The regulation of the system is not yet clear, but since pleiotropic negative mutations occur near both the enzyme and permease loci, and since constitutive mutations occur at the latter locus (SCHWARTZ 1966), the regulation seems to be different from and more complex than in pneumococcus.

The regulatory gene of the pneumococcal system, in the absence of inducer, acts to repress the function of the structural genes. Deletion of the regulatory gene gives high constitutive levels of all components. Non-inducible mutations correspond to single-site alterations or, at most, to small deletions. Since they do not interfere with repression but only prevent induction, the product of such mutant regulatory genes could be a modified, but active, repressor that is unable to interact with and be negated by the inducer. A constitutive mutation overrides the effect of a noninducible mutation in the same gene. These results are most simply and consistently explained by the scheme of negative control proposed for the *lac* system of *E. coli* (JACOB and MONOD 1961), in which the normal regulator gene produces a cytoplasmic repressor that, in the absence of inducer, acts on the structural genes to impede synthesis of the components of the pathway.

The pneumococcal system differs from the *lac* system, however, in several features. The repressed level of synthesis is high, ~ 3% of the induced level, rather than ~ 0.1% as in the *lac* system. In this respect the system parallels those o^c mutants of *E. coli* that constitutively produce β-galactosidase at 1–10% of the level to which they can be induced (JACOB and MONOD 1961). The high basal level in pneumococcus probably serves to allow sufficient initial growth for the cells to adapt to the new sugar. Unlike Enterobacteria, pneumococci cannot metabolise oxidatively. They are restricted, therefore, to sugar fermentation, and on transfer to a new sugar they cannot temporize with energy derived from the oxidative metabolism of other organic substrates.

Another feature of the *mal* system is that coordinate induction of nonadjacent genes is accomplished by negative control. This has recently been shown to be true, also, for the regulation of glycerol catabolism in *E. coli* (COZZARELLI, FREEDBERG and LIN 1968). Negative control in the coordinate repression of nonadjacent genes had previously been demonstrated (MAAS and CLARK 1964). Thus, the type of positive control found in the *ara* system of *E. coli*, where the regulatory gene produces a cytoplasmic activator (SHEPPARD and ENGLESBERG 1967), is not essential for the coordinate induction of a genetically unlinked system.

The amylomaltase and phosphorylase genes appear to form an operon subject to control by an operator region to the left of gene *M*. The absence of phosphorylase in deletions extending to the left of *M* could be due to the deletion of a promoter element (JACOB, ULLMAN and MONOD 1964). Since the deletion *509* terminates within the *D* gene, however, some synthesis of phosphorylase, controlled

by the promoter of the *D* gene, might be expected in this mutant. But the absolute level of such synthesis may be low and not detectable, or else the *D* gene may be transcribed in the opposite direction to that of the *mal-MP* operon.

No clear evidence for polar effects of nonsense mutations was found. Since an appreciable proportion of single-site mutations and a majority of internal deletions are expected to generate nonsense codons of the type found in *E. coli* and its phages (BRENNER and STRETTON 1965), at least some *M* mutants should contain nonsense codons. The absence of effects on the distal gene *P* suggests either that gene *M* is not the first gene in the operon, since polarity effects of nonsense mutations in internal genes can be quite weak (YANOFSKY and ITO 1966; FINK and MARTIN 1967) or that pneumococci, like certain strains of *E. coli*, normally suppress nonsense mutations.

The function of the genetic region in which mutation *V11* occurs is not clear. It does not seem likely that the *V11* mutation alters the interaction of an operator region with the cytoplasmic repressor, for the level of operon function is not enhanced in the context of a constitutive regulatory gene mutant, in which the repressor is presumably absent. The mutation could represent deletion of a strong promoter element and replacement by a weaker promoter. Alternatively, it may generate a nonsense codon in an initial gene of the operon; such a codon could have a strong polar effect on genes *M* and *P*.

Six genes—coding for three permeases, two enzymes, and a cytoplasmic repressor—have been implicated, so far, in the maltosaccharide system. Another structural gene may be altered in the *V11* mutant. Since phosphorylase is not essential for oligosaccharide breakdown, an alternative enzyme for such breakdown may be specified by yet another gene. Furthermore, several mutations at two loci not linked either to each other, or to either the *B* gene or the *mal* region, have also been obtained; these mutants can grow for several generations in maltose medium, but they were unable to form colonies. A complete understanding of the system for maltosaccharide utilization in pneumococcus, therefore, remains to be realized.

I am grateful for the excellent assistance rendered by K. CARLSON, B. GREENBERG, and M. RUDERT in this work.

SUMMARY

Maltosaccharide utilization in pneumococcus involves at least five cellular components. Synthesis of these components is coordinately controlled by the product of a regulatory gene. Separate permeases are responsible for transporting maltose, maltotriose, and maltotetraose into the cell. Two enzymes, amylomaltase and phosphorylase, participate in the catabolism of these sugars, but only the amylomaltase is essential for their utilization.—Mutations affecting the system were mapped by transformation. The genes determining amylomaltase and phosphorylase lie adjacent to each other and form an operon. The regulatory gene and the gene for maltotetraose permease are loosely linked to, but not part of, this operon, while the genes for the other permeases lie elsewhere.—Maltose normally induces high levels of synthesis of amylomaltase and phosphorylase.

Certain mutations of the regulatory gene, however, prevent induction of all five components of the pathway. Other mutations of the regulatory gene, give constitutively high levels of synthesis. Thus, the system for maltosaccharide utilization appears to be under the negative control of a product of the regulatory gene, which, in the absence of induction, impedes synthesis of all components of the pathway.

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