Isolation and Characterization of Catabolite Repression Control Mutants of Pseudomonas aeruginosa PAO

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Independently controlled, inducible, catabolic genes in Pseudomonas aeruginosa are subject to strong catabolite repression control by intermediates of the tricarboxylic acid cycle. Mutants which exhibited a pleiotropism of catabolite repression control of multiple pathways were isolated. The mutations mapped in the 11-min region of the P. aeruginosa chromosome near argB and pyrE and were designated crc. Crc− mutants no longer showed repression of mannitol and glucose transport, glucose-6-phosphate dehydrogenase, glucokinase, Entner-Doudoroff dehydratase and aldolase, and amidas when grown in the presence of succinate plus an inducer. These activities were not expressed constitutively in Crc− mutants but exhibited wild-type inducible expression.

Pseudomonas aeruginosa utilizes a variety of carbohydrates whose initial catabolism by specific transport systems coupled to enzymes leads to the formation of the central metabolite, 6-phosphogluconate (9). Products from the central pathway are then oxidized by the constitutively expressed tricarboxylic acid (TCA) cycle (Fig. 1). The carbohydrate transport systems and the central pathway enzymes are inducible, and their expression is subject to strong repression when acetate or TCA cycle intermediates are present in the growth medium with the sugar (7, 13). The repression of carbohydrate catabolic pathways by these organic acids enables P. aeruginosa to preferentially utilize TCA cycle intermediates before carbohydrates. Thus, when growth medium includes a limited amount of succinate plus an excess of sugar, P. aeruginosa cultures exhibit a diauxic growth response curve that reflects catabolite repression control of inducible catabolic genes (11).

Other inducible catabolic pathways not involved in carbohydrate metabolism are also repressed by growth in the presence of TCA cycle intermediates. Components of these other pathways include amidase (24, 25), alyksulfatase (2, 3), histidase (17), urocanase (18), protocatechuic acid, 3,4-dioxogenase (27), and choline transport (22).

Unlike the glucose effect on catabolite repression found in Escherichia coli and related facultative anaerobes, catabolite repression control of inducible catabolic pathways in P. aeruginosa and Pseudomonas putida does not appear to involve a cyclic-AMP-mediated mechanism (17, 23). While the general phenomenon of catabolite repression in Pseudomonas spp. has been well documented, nothing is known about the molecular basis of this central metabolic control.

This report describes the first isolation and characterization of P. aeruginosa mutants that are defective in catabolite repression control of independently inducible regulatory units that encode pathways for the utilization of both carbohydrates and noncarbohydrates. (Major portions of these results are from the M.S. thesis [25a] and Ph.D. dissertation [25b] of J.A.W.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, phage, and plasmids used in this study are listed in Table 1. Basal salts medium (BSM) contained 50 mM KPO4 (pH 7), 15 mM (NH4)2SO4, 1 mM MgCl2, and 2 μM FeSO4. Carbon sources were used at a final concentration of 20 mM unless otherwise indicated. Amino acid supplements were used at 0.5 mM. All other growth was on Luria broth complex medium (12). Antibiotics were used at the following concentrations: tetracycline, 100 μg/ml; carbenicillin, 500 μg/ml; kanamycin, 500 μg/ml; nalidixic acid, 1 mg/ml. Cells were grown at 37°C, and growth was measured in a Klett-Summerson colorimeter with a no. 66 filter.

Mutagenesis. Mutagenesis with ethyl methanesulfonate (EMS) was carried out by the addition of 200 μl of EMS to cells suspended in 20 ml of carbon-free BSM followed by incubation at 37°C for 60 min. N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis was performed by adding NTG at 100 μg/ml to a mid-log-phase culture growing in Luria broth medium. After 30 s, cells were filtered onto a 0.65-μm-pore-size nitrocellulose membrane and washed three times with 2 ml of BSM. After mutagenesis with either EMS or NTG, cells were allowed to grow overnight in Luria broth medium prior to growth in the enrichment medium.

Enrichment. PRP705 was isolated after EMS mutagenesis of PAO1 by an enrichment scheme based on the method of Zwaag and Lin (26). Cells were grown overnight in succinate-BSM, diluted 1:20 into medium containing 10 mM succinate plus 20 mM mannitol, incubated for approximately 10 h, and finally transferred back to succinate-BSM for overnight growth. After approximately 40 such cycles, the culture was screened for mutants (see below). The spontaneous mutant, PRP701, was also isolated after enrichment in this fashion but without prior mutagenesis.

A second group of mutants, PRP710 to PRP770, was isolated after NTG mutagenesis of PAO1 by 24 h of growth on succinate-BSM containing lactamide as the sole nitrogen source followed by overnight growth on succinate-BSM.

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Table 1. Strains, plasmids, and phage used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>P. aeruginosaa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Prototroph</td>
<td>5</td>
</tr>
<tr>
<td>PAO2</td>
<td>ser-3</td>
<td>B. W. Holloway</td>
</tr>
<tr>
<td>PAO25</td>
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<tr>
<td>PAO303</td>
<td>argB18</td>
<td>4</td>
</tr>
<tr>
<td>PAO483</td>
<td>pyrE</td>
<td>B. W. Holloway</td>
</tr>
<tr>
<td>PAO944</td>
<td>cys-54 pur-67 thr-9001</td>
<td>21</td>
</tr>
<tr>
<td>PAO1632</td>
<td>ami-151 hisC107 hisU108</td>
<td>P. H. Clarke</td>
</tr>
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<td>PAO4171</td>
<td>ilvBC car-9</td>
<td>H. Matsumoto</td>
</tr>
<tr>
<td>PFB105</td>
<td>mtr-2</td>
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</tr>
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<td>PRP701</td>
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</tr>
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<tr>
<td><strong>Plasmids</strong></td>
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</tr>
<tr>
<td>R68.45</td>
<td>Cb' Km' Tc' Cma+ Tra+</td>
<td>4</td>
</tr>
<tr>
<td>pR0271</td>
<td>Cb' Hg' Cma+</td>
<td>15</td>
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<tr>
<td><strong>Phage</strong></td>
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<td></td>
</tr>
<tr>
<td>F116L</td>
<td>B. W. Holloway (8)</td>
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</tbody>
</table>

*a All *P. aeruginosa* chromosomal gene designations are according to Holloway et al. (5).  
*b PRF and PPB strains were derived from strain PAO1.*
were thawed and suspended in 0.1 M Tris (pH 8) and disrupted by ultrasonic oscillation. Supernatant fractions were collected after centrifugation at 105,000 × g for 2 h at 4°C (6).

**Enzyme assays.** Amidase (EC 3.4.1.5) transferase activity was measured in whole-cell suspensions by monitoring the production of acetolhydroxamate from acetamide and hydroxylamine hydrochloride (1). NAD- and NADP-linked spectrophotometric assays for phosphoglucoisomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and glucokinase (EC 2.7.1.2) activities and the combined activity of 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) were performed with cell extracts as previously described (6).

**Other assays.** Uptake of [14C]mannitol and [14C]glucose was measured by a membrane filtration method as previously described (16). Protein was determined by the method of Lowry et al. (10). The concentration of glucose in cell cultures was measured with Glucostat special reagent (Worthington Diagnostics, Freehold, N.J.) by using the procedure described by the manufacturer.

**RESULTS**

**Isolation of Crc- mutants.** Since there is no reliable direct selection for the Crc- phenotype, a method of enriching for Crc- mutants within the total population of a mutagenized culture was required before the culture was screened on plates containing succinate and [14C]mannitol. Two different procedures (see Materials and Methods for details) were used for this enrichment. In the first procedure, a culture was subjected to multiple cycles of growth in BSM containing succinate as the sole carbon source and then in medium containing succinate plus mannitol. The rationale for this was that mutants in which the mannitol pathway was not repressed (i.e., Crc-) would be able to grow faster than the wild type in the presence of mannitol plus succinate as succinate was depleted from the medium. Only two Crc- mutants were isolated after cycles in medium containing succinate plus mannitol: PRP701, a spontaneous mutant, and PRP705, isolated after EMS mutagenesis. When doubling times of these mutant isolates were examined, the mutants were found to grow slightly faster than the wild type in BSM containing succinate or in succinate plus either mannitol or glucose (doubling times were 63 min for PA01, 53 min for PRP701, and 55 min for PRP705). Growth rates on the sugars alone were the same for mutant and wild-type strains (doubling time with glucose was 89 min). Succinate uptake activity, induced by growth on succinate, ranged from 34 to 44 nmol/min/mg of whole-cell protein in all strains. These results demonstrated that the mutants were not impaired for succinate catabolism.

A second enrichment procedure utilized serial subculture in BSM containing succinate as the carbon source and lactamide as the sole source of nitrogen. The rationale in this case was that since amidase (which converts lactamide to ammonia and lactic acid) is repressed by succinate in the wild type (24, 25), growth on lactamide and succinate would be very slow; however, if amidase was not repressed in Crc- mutants, they would grow much more rapidly in the population. Seven independent isolates were selected as presumptive Crc- mutants after this enrichment procedure.

**Induction of mannitol catabolic pathways and transport systems in mutant and wild-type cells.** The mannitol and glucose transport systems and the enzymes of the central carbohydrate catabolism were measured in mutant isolates PRP701 and PRP705 and in wild-type strain PA01. For each assay, cultures growing exponentially in succinate-BSM were sampled and transferred into fresh, prewarmed, aerated medium containing 40 mM succinate and 10 mM mannitol. To maintain optimum balanced growth, cultures were diluted back into fresh succinate-mannitol medium after every other doubling. Figure 2 shows that all of the inducible activities measured were no longer repressed in the mutants. For example, both mannitol transport and glucose-6-phosphate dehydrogenase activity were rapidly induced after cells were transferred to medium containing mannitol, and after 10 generations, these activities increased 20- to 40-fold over those of wild type, whose levels remained close to the noninduced levels. Glucose transport activity also increased 28-fold over the noninduced levels in mutant strains after three generations in medium containing glucose plus succinate. Glucose transport in the wild type increased only threefold during this time (data not shown). On the other hand, phosphoglucoisomerase, a constitutive enzyme not subject to catabolite repression control, showed no significant difference among all three strains.

**FIG. 2.** Effect of crc mutations on expression of carbohydrate catabolic enzymes. At generation 0, cultures growing exponentially in BSM containing 40 mM succinate were sampled and transferred to fresh medium containing succinate supplemented with 10 mM mannitol. At each subsequent generation, additional samples were removed, and at every other doubling, the cultures were transferred to fresh medium containing succinate plus mannitol. Cultures were sampled at 1, 2, 3, 5, and 10 generations after transfer to medium containing mannitol. Mannitol uptake (in nanomoles per minute per milligram of protein), glucose-6-phosphate dehydrogenase (in milli-International units per milligram of protein), phosphoglucoisomerase (in milli-International units per milligram of protein), and Entner-Doudoroff dehydratase and aldolase (in milli-International units per milligram of protein) activities were measured in each sample (as described in Materials and Methods). Each value varied by less than 10% from the mean of three or more determinations. Symbols: ●, PA01 (wild type, Crc+); ■, PRP705 (Crc-); ▲, PRP701 (Crc-).
Finally, Entner-Doudoroff enzyme activities were 2.5-fold higher in the mutants than in the wild type, whose levels again remained at the noninduced level. Thus, inducible enzymes which were repressed by succinate in wild-type cells were not repressed in either mutant, and induction occurred immediately after exposure to inducer.

Diacysic growth. The inability of _P. aeruginosa_ to utilize sugars during growth in the presence of succinate is well documented (9). Because of the repression of the sugar catabolic enzymes by succinate and other TCA cycle intermediates, the depletion of succinate from a culture growing in medium containing succinate plus a sugar results in a lag period of no growth followed by a period of slower growth as the cells shift to catabolism of the sugar. This phenomenon is referred to as diauxie. Does the Crc⁻ phenotype, which allows induction of sugar catabolic pathways in the presence of succinate, abolish this diauxie effect? To answer this question, mutant and wild-type strains were grown in 6 mM succinate plus 14 mM glucose, and the time course of induction of the glucose catabolic enzymes relative to the depletion of succinate in the medium was monitored. Figure 3A shows that wild-type cells utilized succinate exclusively until it was exhausted and then shifted to catabolism of glucose. The expression of glucose uptake (Fig. 3A) and the glucose catabolic enzymes glucose-6-phosphate dehydrogenase and glucokinase (Fig. 3B) occurred only after cells stopped growing on succinate. In the mutant, on the other hand, induction of the glucose-induced pathway occurred in the presence of succinate; however, the induction appeared to be biphasic, with an initial slow rate of enzyme and transport expression which increased again after succinate was depleted. In spite of the ability of the mutant to synthesize glucose catabolic enzymes in the presence of succinate, the growth in the mutant culture was also biphasic. When mannitol or fructose was substituted for glucose, mutant and wild-type strains exhibited patterns of biphasic growth similar to those presented in Fig. 3 (not shown).

Lactamide-dependent growth in the presence of succinate. A larger group of Crc⁻ mutants was isolated after enrichment by growth on succinate with lactamide as the sole nitrogen source. Inducible expression of the mannitol uptake activity also was no longer repressed during growth of these mutants in the presence of succinate. To determine whether both enrichment procedures selected for mutations in the same locus, a representative strain isolated by each method was tested for loss of catabolite repression control of amidae. PRP705 (isolated after it was cycled through growth on succinate with and without mannitol) and PRP720 (isolated after growth with lactamide as the sole nitrogen source) were compared with respect to growth on succinate with lacta-
amide as the sole nitrogen source. Figure 4 shows that both \textit{Crc} mutants were able to grow almost as rapidly using lactamide for nitrogen assimilation as on freely available ammonium, while the wild type grew very slowly with lactamide as the sole source of nitrogen. This suggests that both mutants contain a mutation in the same gene or in functionally similar genes which are responsible for catabolite repression of both amidase and mannnitol transport and catabolic genes.

Amidase expression in mutant and wild-type cells. To ensure that growth on lactamide was indeed a reflection of amidase activity, PRP720 and wild-type PAO1 were examined for amidase expression during growth on lactamide plus succinate in BSM with ammonium. Expression of amidase in PAO1 increased very slowly in the presence of succinate, reaching levels only 2- to 3-fold higher than the noninduced value; however, the \textit{Crc} mutant began expressing amidase after one generation of growth, and after three generations, the specific activity was 60-fold greater than the noninduced level (Fig. 5).

Expression of other carbohydrate catabolic enzymes in PRP720. Since PRP720 (isolated following enrichment in medium containing lactamide as the sole nitrogen source) was selected by using the plate assay for [15C]mannitol uptake, this mutant was presumed to be lacking in catabolite repression control of the mannitol pathway. To confirm that the other inducible enzymes of carbohydrate catabolism were also no longer under catabolite repression control, PRP720 was tested for expression of mannitol uptake and these other enzyme activities. The results were very similar to those shown for PRP701 and PRP705 in Fig. 2.

Conjugal mapping of \textit{crc} mutations. The approximate genetic loci of the \textit{crc} mutations were determined by conjugal mapping experiments. Donor strain PRP706 (a \textit{Met} derivative of PRP705) carrying chromosome-mobilizing plasmid pRO271 was mated with recipient strains containing marker mutations located from 1 to 34 min on the PAO chromosome (15). The highest frequency of coinheritance of the \textit{crc}-5 mutation (95\%) was observed with the \textit{argB18} locus at 11 min (Table 2).

Donor strains containing the \textit{crc}-10 through \textit{crc}-70 mutations and carrying chromosomal mobilization plasmid R68.45 were crossed with recipient strains containing marker mutations located from 1 to 66 min. Mapping experiments with these strains showed that the highest linkage of these mutations was to the \textit{argB} locus (Table 3). The sole

\begin{table}[h]
\centering
\caption{Conjugal mapping of \textit{crc}-5 allele by using donor strain PRP706 and pRO271\textsuperscript{a}}
\begin{tabular}{llr}
\hline
Recipient & Selected allele & Frequency of coinheritance of \textit{crc-5}\textsuperscript{c} \\
strain & inheritance\textsuperscript{b} & \\
\hline
PAO4171 & \textit{car}-9 & 0.12 (31/316) \\
PAO303 & \textit{argB18} & 0.95 (323/340) \\
PAO1632 & \textit{hiuU108 hiuC107} & 0.31 (40/127) \\
PAO25 & \textit{argF10} & 0.12 (41/340) \\
\hline
\end{tabular}
\textsuperscript{a} Procedures for chromosomal mobilization using pRO271 are described in Materials and Methods.
\textsuperscript{b} \textit{Crc} was scored by the plate assay for uptake of [15C]mannitol as described in Materials and Methods. Other alleles were scored by growth on BSM containing 20 mM succinate (for Arg\textsuperscript{+} and Car\textsuperscript{+}) or BSM with 20 mM histidine (for \textit{His}\textsuperscript{+}).
\textsuperscript{c} Numbers in parentheses are number of transconjugants that inherited the unselected \textit{crc}-5 allele/number of transconjugants scored.
\end{table}
exception was crc-30, which did not exhibit linkage with any of the loci tested. Thus, mutants isolated after either enrichment procedure appear to be affected at a locus near argB. The spontaneous mutation in strain PRP701 could not be mapped because of its characteristic high rate of reversion.

**Transductional mapping of the crc-5 mutation.** The tight linkage of the **crc** locus with **argB** indicated that these loci might be cotransducible. To determine the location of **crc** relative to **argB**, the frequency of cotransduction of **crc** with **argB** and **pyrE** (11 min) was tested. A lysate containing the generalized transducing phage F116L propagated on PRP705 (crc-5) was used to transduce strains PAO303 (argB18) and PAO483 (pyrE). Transductants (Arg+ or Pyr+, respectively) which grew on BSM were selected, and cotransduction of the unselected donor **crc-5** allele was scored as increased uptake of radiolabeled mannitol in the presence of succinate. The **crc-5** locus was cotransducible at a frequency of 9% (31 of 316) with **argB** and 37% (19 of 51) with **pyrE**. Since the observed frequency of cotransduction of the **pyrE** locus with the **argB** locus was 31% (17 of 55), the relative gene order in this region must be **argB-pyrE-crc**.

**DISCUSSION**

The enrichment schemes used prior to screening and selection of these mutants were designed to circumvent the problem of isolating mutants with reduced rates of succinate metabolism. Both of these enrichment procedures required that mutants grow as well on succinate as the wild type, and both led to the isolation of strains which appeared to have a mutation in the same gene. The evidence for this is that (i) all mutations which could be mapped were located in the 11-min region of the **P. aeruginosa** chromosome, and (ii) activities of multiple, independently regulated pathways in mutants isolated after either enrichment method were no longer cotable repressed during growth in the presence of succinate or other TCA cycle intermediates. This is illustrated by the comparison of several independently regulated catabolic pathways in mutants isolated by each enrichment procedure.

All carbohydrate catabolic enzymes and transport systems tested which were repressed in the wild type by growth in the presence of succinate were no longer repressed in these **crc**-mutants, yet expression of these enzymes was still inducible, not constitutive, in the mutants. These enzymes and transport systems are not all coordinately regulated but belong to separate regulatory units. These include (i) mannitol transport, which, along with the mannitol catabolic enzymes (mannitol dehydrogenase and fructokinase), is induced by growth in the presence of mannitol (9); (ii) the glucose transport system, which is induced by growth in the presence of glucose and is regulated separately from the central pathway enzymes involved in glucose catabolism (7); (iii) the central pathway enzymes (Entner-Doudoroff dehydratase and aldolase, glucokinase, and glucose-6-phosphate dehydrogenase), which are part of the condicube **hex** regulon (7, 9); and (iv) amidas, an enzyme involved in both nitrogen assimilation and carbon metabolism, which is induced by lactamide or other amides such as acetamide and butyramide (1). Phosphoglucoisomerase, which is constitutively expressed in the wild type and not repressed by succinate, was not affected by the **crc** mutation.

Organic acids other than succinate were also tested as carbon sources for repression of catabolic pathways. Those which exerted a strong repression similar to that exerted by succinate include acetate, citrate, α-ketoglutarate, fumarate, and malate. In contrast, neither glutamate (the precursor of α-ketoglutarate), pyruvate (the precursor of acetate), nor lactate caused this same strong repression. We do not know what effector molecule is responding to the presence of these repressing acids, but it is apparently not cyclic AMP.

Thus, we have defined a **crc** locus at approximately 11 min on the **P. aeruginosa** chromosome that is responsible for catabolite repression control of multiple, independently regulated catabolic pathways. As added evidence that a single gene is defective in these mutants, we have recently identified a gene responsible for **Crc** activity on a 2-kb fragment of **P. aeruginosa** chromosomal DNA which restores catabolite repression control to both **crc-5** and **crc-20** mutations (10a).

Although **Crc**-mutants were able to express various catabolic pathways in the presence of succinate, this **crc** mutation did not appear to completely abolish the diauxic growth pattern. Both cell growth and synthesis of catabolic enzymes and transport systems showed biphasic rates of increase as succinate was depleted from the medium. This suggests that the phenomenon of diauxie may involve more than the repression of enzymes and transport proteins necessary for growth on a particular carbon source.

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**TABLE 3. Conjugal mapping of **crc** alleles in mutants isolated after enrichment by lactamide utilization**

<table>
<thead>
<tr>
<th>Recipient strain (complemented allele, min)</th>
<th>PRP70 (crc-10)</th>
<th>PRP720 (crc-20)</th>
<th>PRP730 (crc-30)</th>
<th>PRP740 (crc-40)</th>
<th>PRP750 (crc-49)</th>
<th>PRP760 (crc-50)</th>
<th>PRP770 (crc-50)</th>
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<tbody>
<tr>
<td>PAO4171 (car-9, 1)</td>
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<td>0/102</td>
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<td>100/110</td>
<td>47/53</td>
<td>54/55</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0/53</td>
<td>0/53</td>
</tr>
<tr>
<td>PFB105 (mut-2, 48)</td>
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<td>0/58</td>
<td>ND</td>
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<td>0/29</td>
<td>0/51</td>
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<tr>
<td>PAO944 (pur-67, 66)</td>
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<td>0/51</td>
<td>ND</td>
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*Donor Crc- mutants bearing plasmid R68.45 were mated with recipient strains, and transconjugants were selected for inheritance of the indicated allele by growth on succinate-BSM unless otherwise indicated. Spontaneous Nal' derivatives of each auxotrophic strain were used as recipients, and donor strains were counterselected with nalidixic acid (see Materials and Methods). Transconjugants were scored for coinheritance of the unselected **crc** mutation as described in Materials and Methods.

**Marker alleles on PAO chromosomal map (5).**

**Coinheritance frequency with the following donor strains:**

**a** Donor Crc- mutants bearing plasmid R68.45 were mated with recipient strains, and transconjugants were selected for inheritance of the indicated allele by growth on succinate-BSM unless otherwise indicated. Spontaneous Nal' derivatives of each auxotrophic strain were used as recipients, and donor strains were counterselected with nalidixic acid (see Materials and Methods). Transconjugants were scored for coinheritance of the unselected **crc** mutation as described in Materials and Methods.

**b** Marker alleles on PAO chromosomal map (5).

**c** Coinheritance of the unselected **crc** allele/no. of transconjugants with complemented allele. ND, not done.

**d** Ami** mutants were selected by growth on BSM containing 0.5% acetamide as the sole carbon source.

**e** Mta** transconjugants were selected by growth on BSM with 20 mM mannitol as the sole carbon source.
ACKNOWLEDGMENTS

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