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Positive Regulation of Amidase Synthesis in *Pseudomonas aeruginosa*

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Mutants of *Pseudomonas aeruginosa* were isolated that were acetamide-negative in growth phenotype at 41°C and constitutive for amidase synthesis at 28°C. Two mutants were derived from the magno-constitutive amidase mutant PAC111 (C11), and a third from a mutant that had enhanced inducibility by formamide, PAC153 (F6). The three temperature-sensitive mutants produced amidases with the same thermal stabilities as the wild-type enzyme. Cultures growing exponentially at 28°C, synthesizing amidase constitutively, ceased amidase synthesis almost immediately on transfer to 41°C. Cultures growing at 41°C were transferred to 28°C and had a lag of about 0.5 of a generation before amidase synthesis became detectable. Pulse-heating for 10 min at 45°C of a culture growing exponentially at 28°C resulted in a lag of about 0.5 of a generation before amidase synthesis recommenced after returning to 28°C. Acetamide-negative mutants that were unable to synthesize amidase at any growth temperature were isolated from an inducible strain producing the mutant B amidase PAC398 (IB10). Two mutants were examined that gave revertants producing B amidase but with novel regulatory phenotypes. It is suggested that amidase synthesis is regulated by positive control exerted by gene *amiR*.

Pseudomonas aeruginosa utilizes acetamide and propionamide for growth. These two amides induce the synthesis of an amidase that hydrolyzes a limited range of short-chain aliphatic amides, and the inducer specificity is quite distinct from the substrate specificity of the enzyme (17). Among the aliphatic amides, formamide is a poor inducer, and although it can be hydrolyzed by the enzyme, it provides only a nitrogen source since one-carbon compounds are not assimilated by this species. Constitutive mutants can be isolated by selection on minimal agar plates with formamide as the nitrogen source and succinate as the carbon source, since on this medium constitutive mutants have a growth advantage over the wild-type strain. Other mutants isolated from succinate-formamide medium include some altered in inducer specificity so that they are more readily induced by formamide than the wild-type parent. The four-carbon amide, butyramide, is not an inducer and acts as an anti-inducer, or amide analog repressor, of amidase synthesis, competing with nonsubstrate-inducing amides, such as *N*-acetylacetamide (7, 8). Butyramide also prevents amidase synthesis in certain constitutive strains, and these have, as do the wild type, a butyramide-negative phenotype. Other constitutive mutants are resistant to butyramide repression and synthesize sufficient wild-type A amidase to allow rather slow growth on butyramide, which is hydrolyzed at about 2% of the acetamide rate (10). Brammar et al. (8) showed that constitutivity and formamide inducibility were cotransduced with the acetamide-positive character at frequencies greater than 90%. No linkage was found with any of the genes for acetate metabolism. Later, Betz et al. (2) examined a number of strains which carried mutations in the amidase structural gene resulting in enzymes with altered substrate specificities. The altered enzyme mutations were also cotransduced at high frequencies with mutations conferring constitutivity. These findings indicated that the amidase regulator and structural genes (*amiR, amiE*) were closely linked and were not associated with genes concerned with the further metabolism of acetate. It was not possible to determine at this time whether amidase synthesis was under positive or negative regulation.

The first experiments indicating negative control of the *lac* operon relied on demonstrating transdominance of the inducible I^+ over the constitutive I^- allele (19). Evidence for positive control of the L-arabinose operon is more complex, but an essential part was the dominance of the constitutive allele $araC^c$ over the pleiotropically negative $araC^-$ (12). No stable diploids

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are available for the *P. aeruginosa* amidase system, and since only one structural gene is involved, it is not possible to identify pleiotropically negative mutants. We have obtained mutants which appear to have temperature-sensitive mutations in the amidase regulator gene and present here evidence that suggests that amidase synthesis is under positive regulation. Bloom et al. (6) concluded from several lines of evidence that L-serine deaminase was under positive control. Among their findings were the observations that certain mutants with a negative phenotype, presumed to be due to mutations in the regulator gene, gave rise to a mixture of inducible and constitutive revertants. We have obtained similar findings with some acetamide-negative mutants.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacterial strains used in this investigation with the relevant phenotypes and genotypes. Series numbers are given to amidase mutants to indicate the main characteristics of each class of mutant and their method of isolation, and these designations will be used in this paper for ease of reference. Mutants are lyophilized as soon as possible after isolation and stock cultures are maintained on nutrient agar stabs and subcultured as required for use.

Media and growth conditions. Amide minimal media have been described previously (8, 9). Media used in experiments on kinetics of amidase synthesis are described with the relevant experiments. For overnight growth, cultures were grown in 5 ml of medium in screw-cap bottles, capacity 25 ml. For experiments with exponential cultures, conical flasks were used containing media up to 20% of the flask volume. Cultures were incubated with shaking on a mechanical shaker.

Cell-free extracts. Bacteria were harvested by centrifugation and resuspended in appropriate buffer. The cells were disrupted with an automatic French pressure cell (American Instruments Ltd.) at pressures between 16,000 and 20,000 lb/inch^2.

Amidase assays. The enzymes were assayed by the acyltransferase reaction with acetamide or butyramide as substrates, adjusting the sample volume and the incubation time according to the specific activity (8). The micromethod used to distinguish the B amidase from the wild-type A amidase in transductants and revertants was that devised by Betz (2) and depended on the ratio of activities toward butyramide and acetamide. A amidase has a butyramide/acetamide ratio of <1%, and B amidase has a ratio of 9 to 10%.

TABLE 1. *Strains of P. aeruginosa used*

Strain no.	Series no.	Selection medium[a]	Parent	Amidase phenotype	Amidase genotype	Reference
PAC1	Wild type			Inducible, A amidase	*amiR*$^+$ *amiE*$^+$	17
PAC101	C1	S/F	PAC1	Constitutive, A amidase, butyramide resistant	*amiR1 amiE*$^+$	8
PAC111	C11	S/F	PAC1	Constitutive, A amidase, butyramide sensitive	*amiR11 amiE*$^+$	8
PAC153	F6	S/F	PAC1	Formamide inducible, A amidase	*amiR43 amiE*$^+$	8
PAC142[b]	L10	S/L	PAC1	Constitutive, A amidase, butyramide resistant, catabolite repression resistant	*amiR33 amiE*$^+$	21
PAC351	B6	B	C11	Constitutive, B amidase	*amiR11 amiE16*	9
PAC377	PhB3	S/Ph	B6	Constitutive, Ph amidase	*amiR11 amiE16,67*	2
PAC398	IB10	AM	PhB3 × Am7	Inducible, B amidase	*amiR*$^+$ *amiE16*	2
PAC307	Am7	Neg AM	PAC1	Acetamide negative	*amiR*$^+$ *amiE7*	2, 8
PAC308	Am8	Neg AM	PAC1	Acetamide negative	*amiR*$^+$ *amiE8*	2, 8
PAC322[c]	CAm2	Neg AM	C11	Acetamide negative	*amiR*$^+$ *amiE18*	2, 8
PAC437	RTS1	P/Fl	C11	Ami$^+$ 28°C, Ami$^-$ 41°C	*amiR11,124 amiE*$^+$	This paper
PAC438	RTS21	P/Fl	C11	Ami$^+$ 28°C, Ami$^-$ 41°C	*amiR11,125 amiE*$^+$	This paper
PAC474	RF17	P/Fl	F6	Ami$^+$ 28°C, Ami$^-$ 41°C	*amiR43,221 amiE*$^+$	This paper
PAC623	FIB29	P/Fl	IB10	Acetamide negative	*amiR223 amiE16*	This paper
PAC624	FIB32	P/Fl	IB10	Acetamide negative	*amiR224 amiE16*	This paper

[a] Selection media used were: S/F, 1% succinate–0.1% formamide; S/L, 1% succinate–0.1% lactamide; B, 0.2% butyramide; S/Ph, 1% succinate–0.1% phenylacetamide; P/Fl, 0.4% pyruvate–0.1% ammonium sulfate–0.4% fluoroacetamide; Neg AM, 0.1% acetamide containing 0.002% succinate to select for minute shadowy colonies; AM, 0.1% acetamide.

[b] PAC142 also carried an unlinked mutation conferring resistance to catabolite repression by succinate.

[c] PAC322 was selected from a constitutive strain but regained the inducible regulatory phenotype as well as the mutation in *amiE*.

Specific activities. The bacterial dry weight was calculated from the absorption at 670 nm, and an optical density of 1.0 was taken as equivalent to 0.56 mg of dry weight or 1.4×10^9 viable bacteria per ml. The specific activity is defined as micromoles of acylhydroxamate produced per minute per milligram of dry weight bacteria.

Mutagenesis. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, to give a concentration of 100 μg/ml, was added to a 6-h culture from nutrient broth resuspended in 0.1 M citrate buffer, pH 6.0, incubated for 45 min at 37°C without shaking, and the culture was then centrifuged, resuspended in dilution buffer, and plated immediately on the appropriate selection medium. For some selections, the mutagenized culture was grown overnight in nutrient broth before plating on selective media, and this procedure was always followed for negative selection methods. Ethyl methane sulfonate (EMS) was added as 10 drops to 5 ml of an overnight culture in nutrient broth. The culture was shaken vigorously for about 1 min, then left at room temperature without shaking for 30 min. The mutagenized culture was centrifuged, resuspended in dilution buffer, and plated on selective media either immediately or after overnight subculture in nutrient broth.

For the revertant studies, an overnight culture in nutrient broth was resuspended in dilution buffer and spread on the appropriate plate. A disk of filter paper (soaked in a solution of a mutagen) was placed in the center of the plate; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, EMS, or 2-aminopurine was used as mutagen. In some experiments a crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used instead of the paper disk.

Transduction. The methods were as described by Betz et al. (3), using bacteriophage F116 (15). For some experiments with slow-growing transductants the plates were incubated in plastic bags for up to 11 days at the appropriate temperatures.

Electrophoresis. Starch-gel electrophoresis was carried out as described by Brown et al. (9).

Reagents. Acetamide and butyramide were obtained from British Drug Houses Ltd., and recrystallized twice from ethanol. Formamide was obtained from British Drug Houses Ltd., and kept cool and in the dark to minimize spontaneous hydrolysis. Lactamide was synthesized by P. D. Laverack. Fluoroacetamide was obtained from Ralph Emmanuel Ltd. Hydroxylamine hydrochloride was obtained from Koch-Light Ltd., and recrystallized twice from ethanol. All other reagents were Analar grade or the best available commercial grade.

RESULTS

Some *lac* operon mutants are constitutive at higher growth temperatures (>37°C) and inducible at lower temperatures. The temperature-sensitive regulatory phenotype results from mutations in the *lacI* gene that lead to the production of a thermolabile *lac* repressor (20). We examined several hundred constitutive mutants of *P. aeruginosa* that had been isolated from succinate-formamide, or butyramide, medium at 37°C to see if any were inducible at lower growth temperatures, and none was found. This negative finding led us to consider the classes of temperature-dependent regulatory mutants that might be expected if amidase synthesis were under positive regulation. We considered that if the *amiR* product were required for transcription of the *amiE* gene, then mutations in the *amiR* gene could lead to an amidase-negative phenotype and some of these might be amidase positive at lower growth temperatures.

Isolation of temperature-sensitive regulatory mutants. About 200 acetamide-negative mutants had been isolated previously by selection on pyruvate-fluoroacetamide medium at 37°C (11). When these were reisolated and tested for growth on acetamide, it was observed that some were leaky and grew on the bench at room temperature (20 to 25°C). These were examined to see whether or not the defect could be ascribed to mutations in the amidase structural gene *amiE*. Amidase is relatively thermostable and is unaffected by heating to 60°C for 15 min. This treatment is used routinely as a step in the purification of the wild-type enzyme (9).

Among the mutants derived from the constitutive strain C11 (PAC111) were two that were acetamide negative at 37°C but grew at a reasonable rate on acetamide plates at 30°C. Cultures of these strains, RTS1 and RTS21, grew at the same rate as the parent strain, C11, in lactate minimal medium at 37°C, but whereas C11 produced amidase in this medium at both 30 and 37°C, the RTS strains produced amidase at 30°C only. No differences could be detected in the substrate specificites or the electrophoretic mobilities of the enzymes. Extracts were prepared from cultures grown at 30°C, and the thermal stability of the amidases produced by these two strains was compared with the wild-type A amidase produced by C11. Figure 1 shows that the amidase from the two mutants was as stable as the wild-type enzyme to heating at 58°C. It should be mentioned here that several of the altered enzyme mutants produce amidases that are very thermolabile and yet allow growth at 37°C, so that if the growth defect had been in the enzyme, it should have been apparent (3). The thermal stability of the amidases produced by the mutants was confirmed by heating the cell extracts at about 67°C. At this temperature there is a progressive loss of activity from the wild-type enzyme, and the extracts from strain RTS1 and RTS21 showed similar effects (Fig. 1A). It was concluded that the inability of the two mutants to grow on acetamide plates at 37°C and to synthesize amidase in lactate medium at this temperature could not be due to mutations in the amidase structural gene.

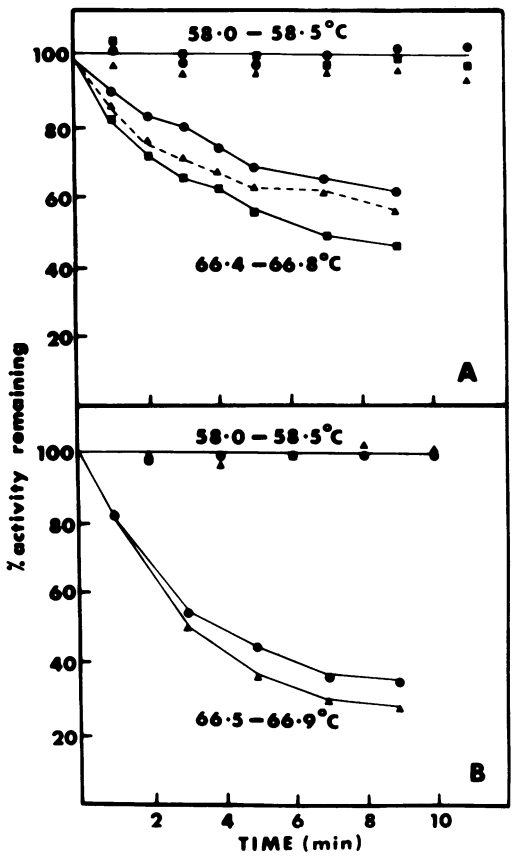

FIG. 1. *Effect of heating on the amidase activities of cell-free extracts of mutants of P. aeruginosa. (A) Constitutive strain C11 (▲), temperature-sensitive mutants RTS1 (■) and RTS21 (●). (B) Formamide-inducible mutant F6 (▲) and temperature-sensitive mutant RF17 (●).*

The two temperature-sensitive regulatory mutants that had been found among the acetamide-negative strains were both derived from a constitutive strain, and it was thought worth attempting to isolate similar mutants from an inducible strain. The wild-type PAC1 was used to select about 100 acetamide-negative mutants from fluoroacetamide plates at 41°C. Most of these were also negative at 30°C, but about 20 grew quite well on acetamide at 30°C but poorly or not at all at 41°C. However none of these strains produced significant levels of amidase in lactate medium, even when lactamide had been added as inducer, and were not investigated further.

We had previously isolated strain F6 which has altered inducer specificity (8). This strain produces wild-type amidase but grows rapidly on formamide-succinate plates because it is readily induced by formamide. Eighteen spontaneous mutants were isolated from pyruvate-fluoroacetamide plates at 37°C. Several of these grew on acetamide plates at 25 but not at 37°C and were found to be constitutive for amidase synthesis, producing higher enzyme levels at 30 than at 37°C. Strain RF17 showed the greatest difference in specific activities at the two temperatures and was investigated in the same way as RTS1 and RTS21. Figure 1B shows that the amidases produced by strains F6 and RF17 responded in the same way to heating, and these results were similar to those obtained previously with RTS1 and RTS21. It was interesting that all the temperature-sensitive mutants derived from the inducible strain F6 were constitutive; the implications of this finding will be considered later.

Another constitutive strain, C1, had been used previously to select acetamide-negative mutants, and of 48 of these mutants examined, 1 strain RTS61 grew on acetamide plates at 30 but not at 41°C. This strain had no amidase activity when grown at 41°C in lactate medium, but the specific activity of cultures grown at 30°C was too low for further studies to be worthwhile.

Rates of amidase synthesis at different temperatures. The previous experiments indicated that the temperature-sensitive lesions of the mutants were more likely to be due to defects in amidase synthesis than to defects in the enzyme protein. The differential rates of amidase synthesis by C11 and RTS1 and RTS21 were measured over a range of temperatures from 20 to 40°C. It can be seen from Fig. 2 that whereas there was no difference in the differential rate of amidase synthesis by C11 at these temperatures, the differential rates for RTS1 and RTS21 declined as the temperature increased above about 25°C. The mean generation times for the three strains are the same and increase from 160 to 180 min at 27°C to 50 to 60 min at 41°C when cultures are grown in lactate medium.

Temperature-shift experiments. It was predicted that cultures of strains RTS1 and RTS21 would lose the ability to synthesize amidase if they were transferred to higher growth temperatures. Figure 3A shows a shift-up experiment in which RTS21 was grown in lactate minimal medium. The parent strain C11 is sensitive to butyramide repression and to catabolite repression by succinate. Both these effects are thought to operate directly, or indirectly, at the level of transcription (10, 21, 22). A culture of RTS21 was grown at 28°C, and at the time indicated, one portion was transferred to 43°C; butyramide or succinate (10 mM) was added to each of two other portions, while one was retained as the control. Figure 3A shows that amidase synthesis fell off rapidly after the cul-

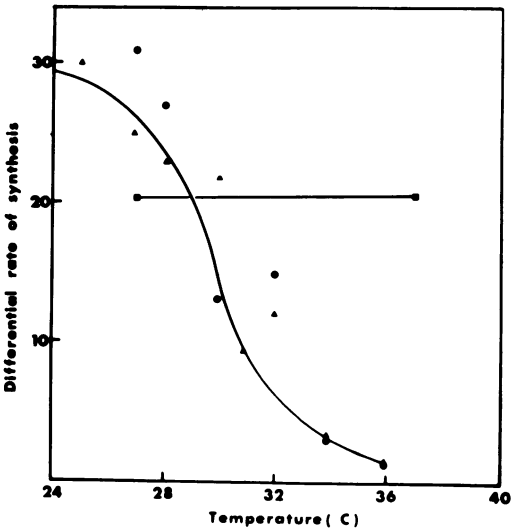

FIG. 2. *Differential rates of amidase synthesis at different growth temperatures in minimal salt medium with lactate (1%) as carbon source. Constitutive strain C11 (■), temperature-sensitive mutants RTS1 (▲) and RTS21 (●).*

ture had been transferred to the higher temperature, and the kinetics of synthesis following temperature shift were similar to the effects of butyramide and succinate on this strain. Similar results were obtained with RTS1 which exhibited the same sensitivities to temperature shift and butyramide and succinate repression (Fig. 3B). RF17 was tested in a comparable experiment and again showed a rapid drop in the rate of amidase synthesis when the culture was transferred from 26 to 43°C. This mutant had been derived from an inducible strain, and at the time of temperature shift, one portion was tested for the effect of an inducing amide. Lactamide did not increase the rate of enzyme synthesis so that RF17 had acquired a mutation conferring constitutivity as well as temperature sensitivity (Fig. 3C).

Experiments in which the temperature-sensitive mutants were transferred from the nonpermissive to the permissive temperatures for amidase synthesis are shown in Fig. 4. It can be seen that for mutants RTS1 and RTS21 there was a lag of about 0.5 of a generation at 28°C before an appreciable rate of amidase synthesis was reached. The lag was less for RF17, and the differential rate of amidase synthesis by this strain at later stages of growth at 43°C was also higher, as expected from the results with overnight cultures.

A further series of experiments was carried out in which cultures were grown at about 28°C and subjected to a period of heat shock by incubating at 45°C for 10 min. It can be seen that with strain RTS21 on return to 28°C there was a lag before the rate of enzyme synthesis recovered to the original level (Fig. 5). *P. aeruginosa* is unable to grow in minimal medium at 45°C so that growth ceases during the heat-shock period, but the cultures recover rapidly when they are returned to the permissive growth temperature.

The constitutive nature of the temperature-sensitive lesion of strain RF17 suggested that some of the constitutive mutants isolated in earlier investigations might exhibit some differences in thermal stabilities. All the constitutive mutants had been isolated at 37°C so that they would not be expected to be thermolabile at this temperature, but it was thought that differences might be detectable after heat shock at higher temperatures. The constitutive strains examined that had been isolated from formamide-succinate medium were C11, parent of RTS1 and RTS21, C1, a butyramide-resistant mutant, and C24, constitutive but repressed by some amides, including *N*-acetylacetamide. Strain L10 was isolated from succinate-lactamide medium and is a constitutive, butyramide-resistant strain that has also an unlinked mutation conferring resistance to catabolite repression by succinate (Table 1). Figure 6 shows that when these strains were transferred from 30 to 42.5°C there were differences between them. The rates of amidase synthesis of C1 and L10 did not change appreciably, but both C11 and C24 had a brief but significant check. This is a very different from the mutants RTS1 and RTS2L in which the shift-up experiment resulted in almost complete cessation of amidase synthesis (Fig. 3). Strain C11 appeared to be more temperature sensitive than the others so it was grown at 28°C and subjected to heat shock for 10 min at 37 and 45°C. As predicted, there was no effect on amidase synthesis of pulse-heating at 37°C, but the culture heated at 45°C had an appreciable lag before it recovered the original rate of amidase synthesis (Fig. 7). These results indicated that selection for constitutive mutants could lead to the isolation of strains producing regulator proteins with different thermal stabilities.

Genetic analysis of temperature-sensitive mutants. It was predicted that the temperature-sensitive mutations of strains RTS1, RTS21, and RF17 would be cotransduced with the *amiE* gene at the usual frequency for *amiR* mutations. In the standard transduction, using bacteriophage F116, the donor phenotype is recovered at a frequency of 2,000 to 3,000/10^9 recipient bacteria, and amidase regulatory mutations are cotransduced with the structural gene at frequencies of about 90%. Betz et al. (2)

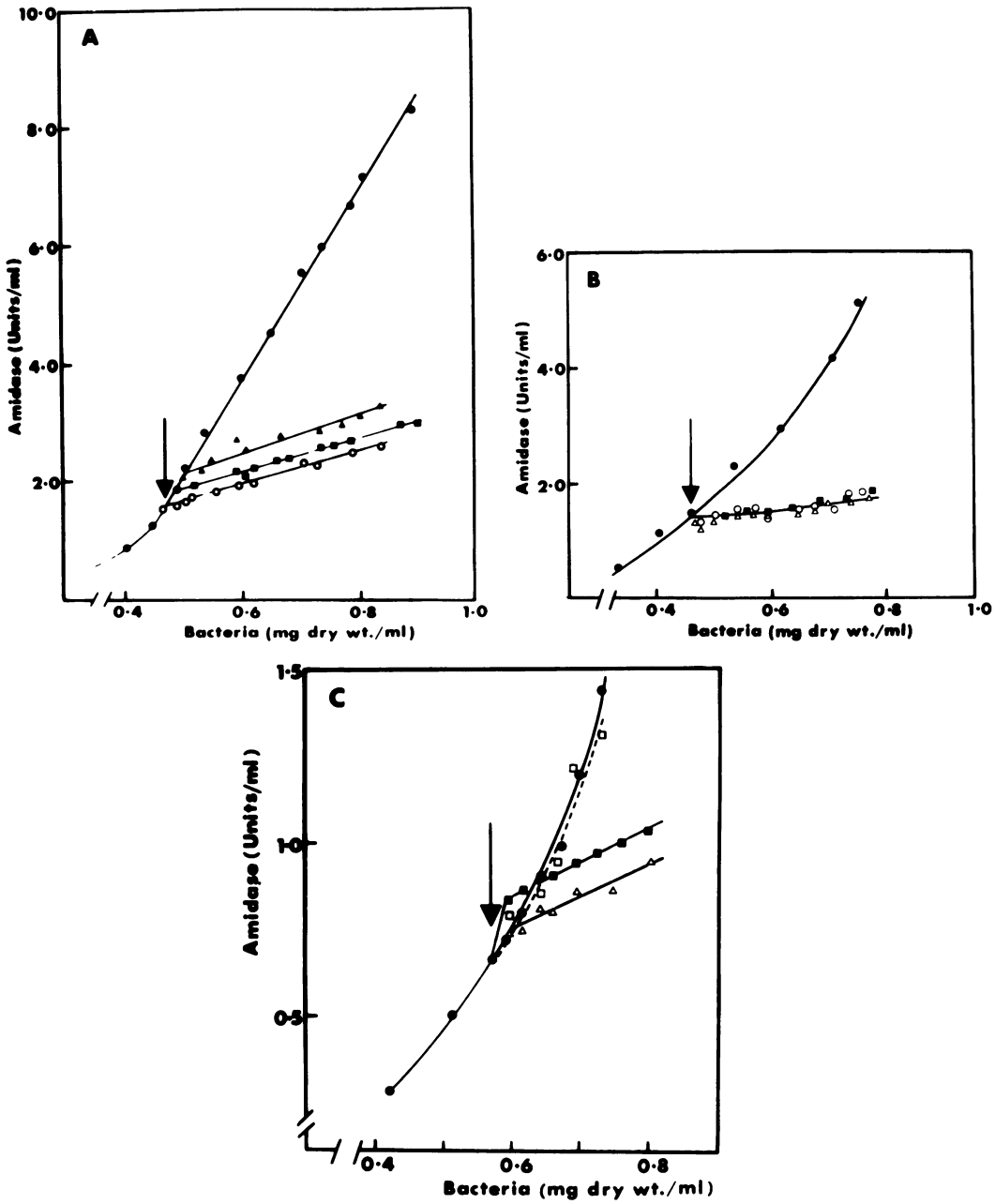

FIG. 3. *Effect of a temperature shift from 28 to 43°C on rates of amidase synthesis by three temperature-sensitive mutants compared with the addition of succinate as catabolite repressor, butyramide as amide analog repressor, or lactamide as inducer. At time indicated by arrow the culture was divided into four parts. One part was transferred to 43°C (■); one part was retained as control (●); and succinate (10 mM) was added to a third part (▲ or △). Butyramide (50 mM) (○) was added to the fourth portion of cultures of RTS21 (A) and RTS1 (B), and lactamide (10 mM) (□) was added to the culture of RF17 (C).*

found that transductants could be recovered as the result of recombination within the *amiE* gene at frequencies ranging from 1 to $100/10^9$ recipient bacteria.

Lysates of bacteriophage F116 were prepared on strains RTS1 and RTS21 and used to transduce the amidase-negative strain Am8 (*amiE8*). After 5 days at 30°C colonies appeared on acet-

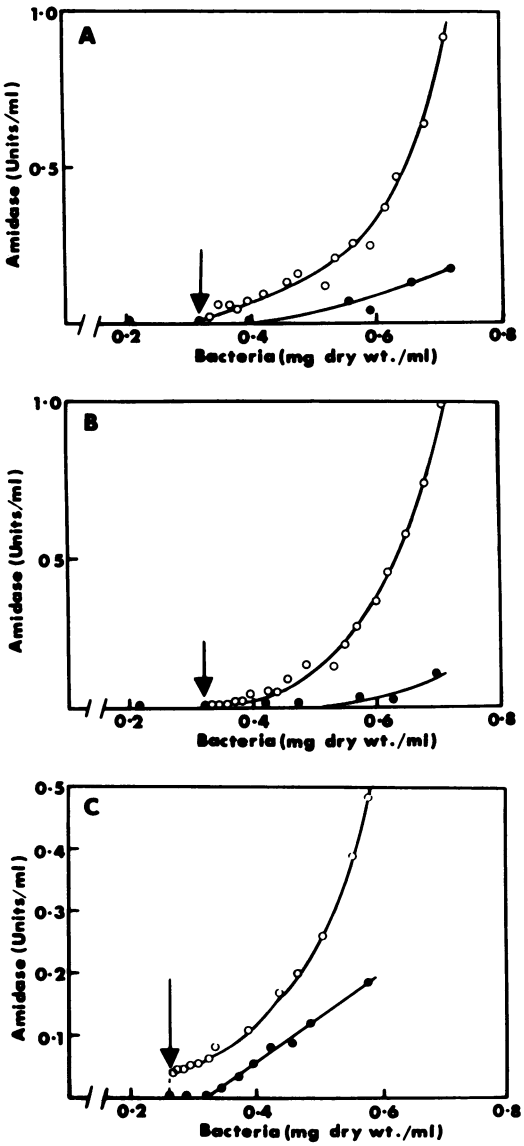

FIG. 4. *Effect of transferring three temperature-sensitive mutants growing at 43°C to the permissive temperature for amidase synthesis, 28°C. At the times indicated by the arrow each culture was split into two parts, one of which was transferred to 28°C* (○) *and the other retained at 43°C* (●). *(A) mutant RTS1, (B) mutant RTS21, and (C) mutant RF17.*

amide plates. These "early" transductants were counted, and the plates were left at room temperature (20 to 25°C) for up to 11 days, by which time more colonies had appeared. The numbers of "early" and "late" transductants obtained in these crosses are shown in Table 2. Some of the early and late transductants were purified by reisolation of single colonies and tested for regulatory phenotype by growing overnight in lactate medium with and without lactamide. The early transductants were all constitutive at both 30 and 41°C, and it was concluded that they had arisen by recombination within the *amiR-amiE* gene region. The late transductants were tested by patching on pyruvate plates and assaying the resultant growth for amidase production by the qualitative semimicromethod (2). Most of the late transductants were constitutive at 30°C and amidase negative at 41°C, indicating that the temperature-sensitive defect had been cotransduced with the amidase structural gene at a frequency of 95%. The recovery of late transductants in these crosses was around $2{,}000/10^9$ recipient bacteria, which is in the range expected for recovery of the donor phenotype. Recombinants could also be recovered in such a cross by selecting on lactamide plates at 41°C. Lactamide is a very poor substrate, and background growth is eliminated. Strain RTS1 was the donor and Am7 (*amiR*$^+$ *amiE7*) the recipient in a cross in which 300 recombinant transductants were selected on acetamide plates at 30°C and 250 on lactamide plates at 41°C (M. Day, personal communication). The transductants recovered at 41°C from the lactamide plates were all similar in phenotype to the early acetamide transductants and were constitutive at both 30 and 41°C. The reciprocal crosses with RTS1 and RTS21 as recipients could not be carried out on acetamide plates because of the background growth of these strains on prolonged incubation both at 37 and 41°C, and no further experiments were done using lactamide medium.

Mutant RF17 could not be used as a recipient in transduction because it had a high reversion rate, but it was quite suitable for propagation of bacteriophage F116 and was used as donor in crosses with several different amidase-negative mutants. The relative positions of mutations *amiE8,18,7* had been established previously (2), and the strains carrying these mutations were used in transductions with RF17 as donor. When selection was carried out on acetamide plates at 25°C, the number recovered was around $2{,}000/10^9$ recipient bacteria, as expected for the transfer of donor phenotype. When the transduction was carried out at 37°C, the transductional frequency was 100 to $300/10^9$ recipient bacteria, as expected for recombination within the amidase genes. The transductants from the cross at 37°C were analyzed for regulatory phenotype after purification from any background growth. In these crosses there was no visible background growth on acetamide plates at 37°C. Transductants were grown at 37°C in lactate medium with and without lactamide as inducer. Table 3 shows that most of the recombinants in

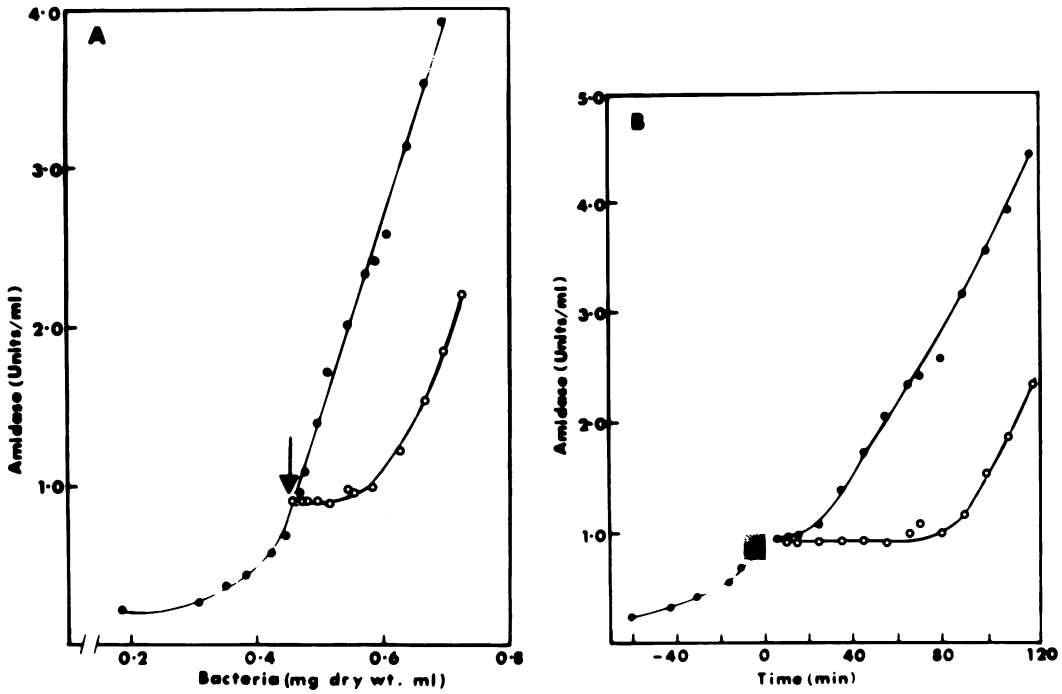

FIG. 5. *Effect of pulse-heating on rate of amidase synthesis of temperature-sensitive mutant RTS21. The culture was grown at 28°C, and at the time indicated by the arrow was divided into two parts, one portion of which was heated at 45.5°C for 10 min, returned to 28°C, and allowed to grow (○); the other portion was kept at 28°C without shaking during this period (●). The differential rates of amidase synthesis are shown in (A) and the time course of the experiment in (B).*

the three crosses were wild-type inducible; none were formamide inducible, and some were constitutive. The relative numbers of constitutive transductants recovered, together with the absence of any formamide-inducible transductants, suggests that the order of the mutations in RF17 is *amiR43,221 amiE* (Fig. 8).

The recovery of a thermostable constitutive recombinant in the crosses between RF17 and the amidase-negative mutants suggested that the second mutation, *amiR221,* determined constitutivity and not thermolability. This indicates that the thermolabile phenotype resulted from the presence of both *amiR43,* formamide inducibility, and *amiR221,* constitutivity, in the same molecule.

Acetamide-negative strains with regulator gene mutations. If the *amiR* gene product is required for transcription of the *amiE* gene, then it can be predicted that some of the mutants isolated as acetamide negative will have defects in the regulatory gene rather than in the amidase structural gene (1). Englesberg and colleagues (12) had obtained a number of *araC*$^-$ mutants which were pleiotropically negative for the L-arabinose enzymes, and Bloom et al. (6) identified *dsdR* mutants that were negative for serine deaminase synthesis. Since only one structural gene appeared to be concerned in the amidase system, we designed experiments on the lines of those of Bloom et al. (6). In such an analysis it is important to be certain that the mutations examined are not in the structural gene for the enzyme, and to ensure this it is necessary to label the structural gene in such a way that it can be identified in revertants. We were able to do this by using a mutant with a mutation in the *amiE* gene that determines the B amidase which can be easily distinguished from the A amidase by its altered substrate specificity (9). Strain IB10 had been obtained as a recombinant in a cross between the phenylacetamide-utilizing mutant PhB3 and the amidase-negative mutant Am8. IB10 is *amiR*$^+$ and is induced by acetamide but carries *amiE16* and produces the B-type amidase. It is unable to grow on butyramide because butyramide cannot induce, but the cultures grown on acetamide can be assayed for substrate specificity by the amidase microtest (2).

A series of mutants was isolated from strain IB10 on pyruvate-fluoroacetamide plates at 37°C. Some arose spontaneously, and others were obtained by mutagenesis with EMS or 2-

aminopurine. Of 41 FIB mutants isolated in this way, 16 were purified and tested for growth phenotype. All were acetamide negative, and 8 gave no revertants either spontaneously or in response to EMS mutagenesis. The strains that gave rise to revertants were examined in more detail, and the revertant colonies were reisolated and tested for regulatory phenotype. Table 4 shows that three classes of FIB mutants could be distinguished. The first class, exemplified by FIB1 and FIB14, gave only revertants with the wild-type-inducible phenotype. It was concluded that the mutations to give the acetamide-negative phenotype had been in the *amiE* gene. The second class, including FIB2, FIB15, and FIB28, gave only constitutive mutants. It was thought that these might have arisen from acetamide-negative mutants with *amiR* mutations, but these were not studied in detail. The third class included mutants FIB29, FIB30, and FIB32. The revertants obtained from these strains comprised a mixture of regulatory phenotypes and were thought to be comparable to those obtained for D-serine deaminase (6).

Fifty-one revertants selected from acetamide

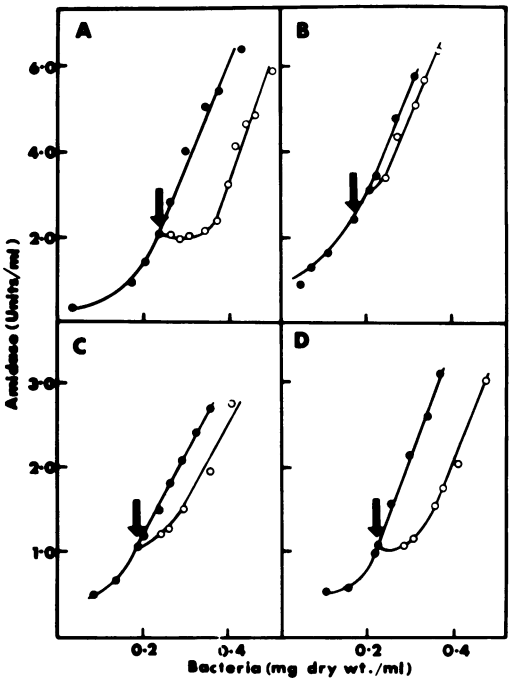

FIG. 6. *Effect on rates of amidase synthesis of transferring four amidase-positive constitutive strains from 30°C, control cultures (●) to 43°C (○). Amidase synthesis continues, but differences in the effect of the shift-up in temperature can be seen between (A) C11, (B) L10, (C) C1, and (D) C24.*

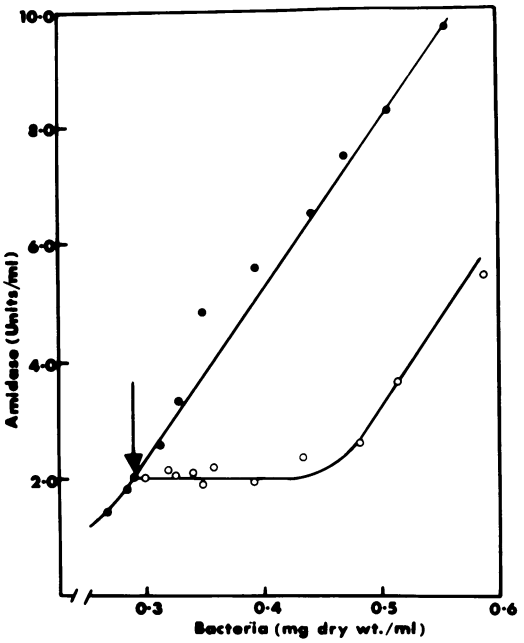

FIG. 7. *Effect of pulse-heating at 45°C on amidase synthesis by the constitutive strain C11. The culture was grown at 28°C, and at the time indicated by the arrow was divided into two portions, one of which was heated at 45°C for 10 min, returned to 28°C, and allowed to grow (○). The other portion was kept at 28°C without shaking during this period (●).*

TABLE 2. *Transduction between strains RTS1 (PAC437) and RTS21 (PAC438) and the amidase-negative strain Am8 (PAC308)*

Donor lysate	Recipient strain Am8 transductants										
	Early (<5 days)					Late (<11 days)					
	No./10^9 recipients	No. of colonies tested	No. constitutive at:		% Thermostable recombinant phenotype	No./10^9 recipients	No. of colonies tested	No. constitutive at:		No. with no activity	% Thermosensitive donor phenotype
			30°C	41°C				30°C	41°C		
RTS1	110	30	30	30	100	2,140	74	72	2	2	95
RTS21	110	18	18	18	100	2,350	85	83	2	2	95

TABLE 3. *Transduction between strain RF17 (PAC474) and acetamide-negative strains Am7 (PAC307), AM8 (PAC308), and CAm2 (PAC322)*

Recipient strains	Donor lysate RF17 (no. of transductants/10^9 recipient bacteria)					
	Total at:		No. of 37°C transductants tested	No. constitutive	No. lactamide inducible	No. formamide inducible
	25°C	37°C				
Am7	2,800	316	44	8	36	0
Am8	1,960	78	35	6	29	0
CAm2	1,250	123	37	8	29	0

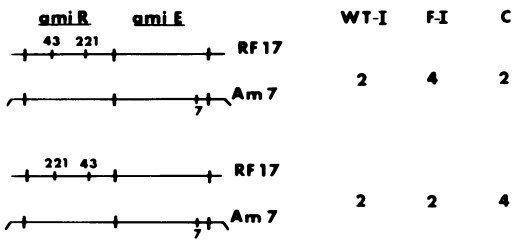

FIG. 8. *Number of crossovers required to produce recombinants in the transductional cross between RF17 as donor and Am7 as recipient. Recombinants predicted are wild-type inducible (WT-I), formamide inducible (F-I), and constitutive (C).*

plates from strain FIB29 were reisolated and tested for enzyme specificity and regulatory phenotype. All of the revertants produced amidases which gave a ratio of activity towards acetamide and butyramide that is characteristic of the B-type enzyme. This finding made it improbable that the acetamide-negative mutation had been due to a mutation in the *amiE* gene, although it did not rule out this possibility. Extracts of seven of the FIB29 revertants were analyzed by starch-gel electrophoresis and gave the characteristic mobility of the B enzyme. Twenty-four of the FIB revertants were tested in more detail and were grown overnight in 1% lactate medium. Lactamide (20 mM) was added to another set of tubes to check whether any of the revertants were inducible. Table 5 gives the numbers and the percentage of the revertants falling into each class. Measurement of specific activities after overnight growth gives an indication of regulatory phenotype, and an arbitrary value of 3.0 was chosen to discriminate between "high" and "low" constitutive strains. A similar experiment was carried out with revertants from FIB32 and of 80 revertants examined, all but one gave a butyramide/acetamide ratio of 8 to 10%, indicating the presence of B enzyme. The proportions of inducibles, and high and low constitutives, were somewhat different, but it must be emphasized that these simple experiments can give only rough comparisons. The revertants were also examined for their sensitivities to catabolite repression by succinate and repression by butyramide. There were also differences in the ways in which the revertants responded in these media. Table 6 gives a few examples of the values obtained. FIB29.5 was a low constitutive which appeared insensitive to both butyramide and succinate repression. FIB29.6 was inducible and repressed by succinate, and FIB 29.35 was a low constitutive repressed by both butyramide

TABLE 4. *Growth of acetamide-positive revertants of FIB mutants on amide selective media*

Series no.	Mutagen	No. of revertants tested	Growth phenotypes of revertants[a]: no. growing on amide media				
			AM	S/F	B	S/B	S/L
FIB1	EMS	5	5	0	0	0	(5)[b]
FIB14	EMS	11	11	0	0	0	(11)
FIB2	EMS	15	15	15	15	15	15
FIB15	EMS	7	7	7	7	7	7
FIB28	EMS	5	5	5	5	5	5
FIB29	Sp[c]	51	51	38	38	38	38
FIB30	EMS	11	11	10	10	10	(10)
FIB32	Sp	20	20	9	9	9	NT

[a] The FIB mutants derived from strain IB10 were tested for reversion on acetamide. Some of the revertant colonies were patched on succinate plates and replicated onto the amide selective media. Media were as given in Table 1, with the addition of 1% succinate–0.1% butyramide (S/B). Plates were incubated 36 h at 37°C.

[b] Numbers in parentheses indicate that only trace growth occurred on this medium; NT, not tested.

[c] Sp, Spontaneous.

TABLE 5. *Regulatory phenotypes of acetamide-positive revertants isolated from mutants FIB29 (PAC623) and FIB32 (PAC624)*

Determination	FIB29 revertants		FIB32 revertants	
	No.	%	No.	%
Inducible[a]	5	21	6	8
High constitutives[a]	9	38	52	65
Low constitutives[a]	10	41	22	27

[a] All activities were measured by the amidase transferase assay with acetamide as substrate for cultures grown overnight in minimal medium with lactate (1%) as carbon source in the presence and absence of lactamide (20 mM) to induce amidase synthesis. High constitutives had specific activities of >3.0 U/mg of bacteria.

TABLE 6. *Amidase activities of acetamide-positive revertants of strain FIB29 (PAC623) grown overnight in lactate and succinate media*

No. of FIB29 revertants	Amidase activities (U/mg of dry wt bacteria[a])			
	1% Lactate medium			1% Succinate medium
	No addition	Lactamide (20 mM)	Butyramide (16 mM)	No addition
5	0.40	0.37	0.36	0.43
6	0.68	2.62	0.49	1.47[b]
35	1.40	1.75	0.72	0.63
33	4.1	3.0	2.2	3.5
37	4.3	3.6	1.6	16.4

[a] All activities were measured by the amidase transferase assay with acetamide as substrate.

[b] Lactamide (20 mM) was added to all inducible cultures grown in succinate medium.

and succinate. FIB29.33 was a high constitutive repressed by butyramide and partially repressed by succinate, whereas FIB29.37 was repressed by butyramide, and its activity was greatly enhanced by growth in succinate. This finding was unexpected since mutations in the *amiR* gene were not expected to alter catabolite repression, but three other FIB29 revertants showed a similar response to succinate as did some of the FIB32 revertants. However, the succinate activation effect observed after overnight growth of revertant FIB29.37 in succinate medium was not reflected in any enhancement of the rate of amidase synthesis when succinate was added to cultures growing exponentially in lactate medium.

Revertants were also isolated from the mutant RF17, and of nine isolates from acetamide plates at 37°C, four were constitutive and the rest inducible. These studies on the regulatory characteristics of revertants isolated from three strains thought to have mutations in the *amiR* gene are similar to those with D-serine deaminase (6) and provide additional evidence for positive regulation of amidase synthesis.

DISCUSSION

The selection methods that proved successful in isolating mutants producing amidases with altered substrate specificities indicated that the classes of structural gene mutants isolated depended on the previous mutations in the parent strain and the selection media. No altered enzyme mutants were obtained directly from the wild-type strain, and in some instances it proved easier to isolate mutants producing enzymes with novel activities from strains that already had at least one structural gene mutation (2, 3). The classes of regulatory mutants that have been isolated were also dependent on the selection medium and the parent strain. Constitutive mutants are readily isolated from succinate-formamide medium and at a lower frequency from butyramide medium. This is partly a technical difference since the background growth on succinate-formamide plates is greater, thus giving a higher apparent frequency. However, there is a real difference as well, since constitutive mutants growing on butyramide must be high constitutives and resistant to butyramide repression, whereas mutants isolated from succinate-formamide medium include high and low constitutives, butyramide-sensitive and butyramide-resistant constitutives, as well as formamide-inducible mutants. Since constitutive amidase mutants can be isolated in large numbers, it was significant that none have been found to be inducible at lower growth temperatures even when a deliberate attempt was made to isolate them. It is easy to isolate large numbers of acetamide-negative mutants from pyruvate-fluoroacetamide medium, many of which are leaky and may have mutations in the structural gene. A detailed examination of leaky mutants identified RTS1 and RTS21, isolated from the constitutive strain C11, and RF17, isolated from the formamide-inducible strain F6. All three mutants produced amidases that were as thermostable as that of the wild type, and the transductional crosses showed that the temperature-sensitive mutations could be located in the *amiR* gene. The recovery of a temperature-sensitive constitutive recombinant in the cross between RF17 and Am7 showed that a single mutation in *amiR* could give this phenotype as well as the two mutations of RTS1 and RTS21. If the *amiR* product is required for transcription, then the mutants with the phenotype of RTS1, and the other two strains, must produce a protein with activity over a well-defined temperature band. For the mutant to be recognized it must grow reasonably well on acetamide plates at 20 to 25°C and poorly or not at all at 37 to 41°C. For detailed studies to be made it is essential for a reasonable amount of amidase to be synthesized at the permissive temperature. This was not the case for the mutants derived from PAC1 or from the constitutive strain C1 so that it is not known whether these strains could give rise to temperature-sensitive regulator mutants using this selection procedure.

Temperature-sensitive mutants of the *lacI*(Ts) class, growing at a temperature at which they were inducible, became constitutive when they were transferred to a higher growth temperature; heating for a short time at 45°C resulted in constitutive β-galactosidase synthesis for about 0.5 generation after they were returned

to the lower temperature. The steady state specific activity of the *lacI*(Tl) mutants increased progressively with increased growth temperature, and the inactivation of the repressor was irreversible (20). These findings were consistent with the interpretation that the *lacI*(Tl) mutants produced thermolabile repressors. The other class of mutants *lac*(Tss) were temperature sensitive for the assembly of the repressor from its subunits, and pulse-heating had no immediate effect on β-galactosidase synthesis in these strains. It was necessary to grow the *lacI*(Tss) mutants at elevated temperatures to obtain the constitutive phenotype. The immediate response of the amidase mutants, RTS1, RTS21, and R17, to a shift-up in temperature and the time taken to recover from the inactivation of amidase synthesis after pulse-heating at 45°C indicate that the temperature-sensitive lesion in these mutants is probably due to a thermolabile activator protein rather than to any defect in synthesis or assembly. Two classes of temperature-sensitive *araC* mutants were described (16). The first class, *araC*(Ts), was inducible at 28°C and Ara⁻ at 42°C, and the second class, *araC*^{c}(Ts), was constitutive at 28°C and Ara⁻ at 42°C and thus resemble the amidase mutants, RTS1, RTS21, and RF17 in phenotype.

The second line of evidence for the positive regulation of amidase synthesis is based on the properties of the revertants from certain acetamide-negative strains. The presence of the *amiE16* mutation in the starting strain IB10 made the identification of the amidase produced by the revertants unequivocal. The mixture of regulatory classes, including inducible strains and high and low constitutives, was similar to that obtained from revertants of the D-serine deaminase *dsdC* strains (6). The mutation producing these revertants need not have been in the regulator gene itself. Some of the *dsdC* revertants were completely insensitive to catabolite repression, and partial diploid tests showed that they were *cis* dominant and therefore in the *dsdO* region. We obtained a large number of revertants from both FIB29 and FIB32 which were altered in their response to catabolite repression and some showed a considerable enhancement of amidase synthesis by succinate. Previously we had isolated promoter mutants that were resistant to catabolite repression and insensitive to the stimulation of amidase synthesis by cyclic AMP shown by the parent strain (22). It is possible that the catabolite repression-resistant mutants isolated from FIB29 and FIB32 were amidase promoter mutants, but this is not the only possible interpretation. The D-serine deaminase system is sensitive to catabolite repression and this can be partially reversed by addition of the inducer D-serine, suggesting that the cyclic-AMP binding protein can be replaced to some extent by the *dsdC* product to which D-serine has been bound (4–6, 18). If this were so, then mutations in the promoter region might restore the ability to make D-serine deaminase in a mutant with a defective *dsdC* product and at the same time result in resistance to catabolite repression. The only evidence we have so far to implicate a similar system for amidase is that (i) catabolite repression can be partially relieved by the addition of inducer (7), (ii) cyclic AMP stimulates amidase synthesis in inducible and constitutive strains (21), and (iii) catabolite repression-resistant mutants have been isolated with properties consistent with being promoter mutants (22).

On the other hand, for the L-arabinose operon some catabolite repression-resistant mutants have been isolated which have mutations in the *araC* gene (14). Transcription of the *ara* genes appears to require a complex comprising the active form of the *araC* gene, cyclic-AMP bound to the receptor protein, together with RNA polymerase. If amidase transcription follows a similar pattern, then it would be reasonable to suppose that mutations in *amiR* could result in resistance to catabolite repression. The L-arabinose operon has a mixed type of regulation with the *araC* product acting as a classical repressor and binding at the operator site, but able to bind the inducer L-arabinose and become converted to the activator form required for transcription. At present we see no reason to postulate a dual control for amidase synthesis and suggest that the *amiR* product acts solely as a positive regulator protein. There have been few reports on the mechanism of regulation of gene expression in *Pseudomonas* species, although there is a wealth of information on inducer specificities and regulatory units. Positive regulation has been suggested for the *catB, catC* genes of *P. putida,* determining two coordinately regulated enzymes of the aromatic ortho-cleavage pathway (24). The TOL plasmids of *P. putida* carry genes for the enzymes of the meta-cleavage pathway and the group of enzymes required for the initial steps for the catabolism of toluene and *m*- and *p*-xylene. Some of these activities can be lost, and the patterns of substrate utilization and inducer specificity suggest that these genes may be under positive control (25, 26).

We suggest that the properties of most of the mutants altered in the regulation of amidase synthesis can be related to changes in the regulator protein specified by the *amiR* gene that

TABLE 7. *Types of mutation in regulatory gene amiR for P. aeruginosa amidase*

Series no.	Phenotype	Gene notation	Reference
PAC1	Wild type	*amiR*$^+$	17
C-series: C11, C24	Constitutive, butyramide sensitive	*amiR*^{c}	8
CB-series and some C-series	Constitutive, butyramide resistant	*amiR*^{c} (b)	10
F-series	Formamide inducible	*amiR* (f)	8
FIB-series: FIB29, FIB32	Regulatory, acetamide negative	*amiR*$^-$	This paper
TS-series: RTS1, RTS21, RF17	Constitutive at 30°C, acetamide negative at 37°C	*amiR*^{c}(Ts)	This paper

exerts positive control on the expression of the amidase structural gene. Table 7 sets out the main types of mutations in the *amiR* gene using a notation based on that applied to *araC* mutations (12, 13, 16). The *amiR*$^+$ protein has an amide binding site that accepts both inducing amides and the amide analog repressing amides, such as butyramide and cyanoacetamide. Mutations such as *amiR*(f) alter the amide binding site in such a way that formamide is more efficient as an inducer. The mutations resulting in constitutive strains may allow amidase synthesis in the absence of an inducing amide without conferring resistance to amide analog repression. These are denoted *amiR*^{c}, whereas those that have also acquired an appreciable resistance to repression by amides such as butyramide are denoted *amiR*^{c}(b). Both *amiR*^{c} and *amiR*^{c}(b) classes can be isolated from succinate-formamide medium, and *amiR*^{c}(b) mutants can also be isolated from butyramide medium. We have now added to thse *amiR*^{c}(Ts), as evinced by the mutants RTS1, RTS21, and RF17 which produce temperature-sensitive but constitutive regulator proteins, and *amiR*$^-$, as evinced by FIB29 and FIB32 with no activity.

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LITERATURE CITED

1. **Beckwith, J., and P. Rossow.** 1974. Analysis of genetic regulatory mechanisms. Annu. Rev. Genet. **8**:1-13.
2. **Betz, J. L., J. E. Brown, P. H. Clarke, and M. Day.** 1974. Genetic analysis of amidase mutants of *Pseudomonas aeruginosa*. Genet. Res. **23**:335-359.
3. **Betz, J. L., and P. H. Clarke.** 1972. Selective evolution of phenylacetamide-utilizing strains of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **73**:161-174.
4. **Bloom, F. R.** 1975. Isolation and characterization of catabolite-resistant mutants in the D-serine deaminase system of *Escherichia coli* K-12. J. Bacteriol. **121**:1085-1091.
5. **Bloom, F. R., and E. McFall.** Isolation and characterization of D-serine deaminase constitutive mutants by utilization of D-serine as sole carbon or nitrogen source. J. Bacteriol. **121**:1078-1084.
6. **Bloom, F. R., E. McFall, M. C. Young, and A. M. Carothers.** 1975. Positive control in the D-serine deaminase system of *Escherichia coli*. J. Bacteriol. **121**:1092-1101.
7. **Brammar, W. J., and P. H. Clarke.** 1964. Induction and repression of *Pseudomonas aeruginosa* amidase. J. Gen. Microbiol. **37**:307-319.
8. **Brammar, W. J., P. H. Clarke, and A. J. Skinner.** 1967. Biochemical and genetic studies with regulator mutants of the *Pseudomonas aeruginosa* 8602 amidase system. J. Gen. Microbiol. **47**:87-102.
9. **Brown, J. E., P. R. Brown, and P. H. Clarke.** 1969. Butyramide-utilizing mutants of *Pseudomonas aeruginosa 8602* which produce an amidase with altered substrate specificity. J. Gen. Microbiol. **57**:273-285.
10. **Brown, J. E., and P. H. Clarke.** 1970. Mutations in a regulator gene allowing *Pseudomonas aeruginosa* 8602 to grow on butyramide. J. Gen. Microbiol. **64**:329-342.
11. **Clarke, P. H., and R. Tata.** 1973. Isolation of amidase-negative mutants of *Pseudomonas aeruginosa* by a positive selection method using an acetamide analogue. J. Gen. Microbiol. **75**:231-234.
12. **Englesberg, E., J. Irr, J. Power, and N. Lee.** 1965. Positive control of enzyme synthesis by gene C in the L-arabinose system. J. Bacteriol. **90**:946-957.
13. **Englesberg, E., and G. Wilcox.** 1974. Regulation: positive control. Annu. Rev. Genet. **8**:219-242.
14. **Heffernan, L., R. Bass, and E. Englesberg.** 1976. Mutations affecting catabolite repression of the L-arabinose regulon in *Escherichia coli* B/r. J. Bacteriol. **126**:1119-1131.
15. **Holloway, B. W., J. B. Egan, and M. Monk.** 1960. Lysogeny in *Pseudomonas aeruginosa*. Aust. J. Exp. Biol. **38**:321-330.
16. **Irr, J., and E. Englesberg.** 1971. Control of expression of the L-arabinose operon in temperature-sensitive mutants of gene *araC* in *Escherichia coli* B/r. J. Bacteriol. **105**:136-141.
17. **Kelly, M., and P. H. Clarke.** 1962. An inducible amidase produced by a strain of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **27**:305-316.
18. **McFall, E.** 1973. Role of adenosine 3',5'-cyclic monophosphate and its specific binding protein in the regulation of serine deaminase synthesis. J. Bacteriol. **113**:781-785.
19. **Pardee, A. B., F. Jacob, and J. Monod.** 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *Escherichia coli*. J. Mol. Biol. **1**:165-178.
20. **Sadler, J., and A. Novick.** 1965. The properties of repressor and the kinetics of its action. J. Mol. Biol. **12**:305-327.
21. **Smyth, P. F., and P. H. Clarke.** 1975a. Catabolite repression of *Pseudomonas aeruginosa* amidase: the effect of carbon source on amidase synthesis. J. Gen. Microbiol. **90**:81-90.
22. **Smyth, P. F., and P. H. Clarke.** 1975b. Catabolite repression of *Pseudomonas aeruginosa* amidase: isolation of promoter mutants. J. Gen. Microbiol. **90**:91-99.
23. **Wheelis, M. L.** 1975. The genetics of dissimilatory pathways in *Pseudomonas*. Annu. Rev. Microbiol. **29**:505-524.
24. **Wheelis, M. L., and L. N. Ornston.** Genetic control of enzyme induction in the β-ketoadipate pathway of

Pseudomonas putida: deletion mapping of *cat* mutations. J. Bacteriol. **109**:790–795.

25. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
26. **Worsey, M. J., and P. A. Williams.** 1977. Characterization of a spontaneously occurring mutant of the TOL20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. J. Bacteriol. **130**:1149–1158.