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Selection for Genetically Repressible (ArgR$^+$) Strains of *Escherichia coli* K12 from Genetically Derepressed (ArgR$^-$) Mutants Using Acetylnorvaline

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Summary. The growth of *E. coli* K12 strains derepressed for N-α-acetyl-L-ornithinase (the *argE* gene product) is inhibited by 25 mg/ml N-α-acetyl-DL-norvaline. This is due to deacetylation of acetylnorvaline by acetylornithinase yielding norvaline which inhibits growth. A genetically repressible (*argR*$^+$) strain (514) in which acetylornithinase is repressed is resistant. Thus, acetylnorvaline resistance can be used to select *argR*$^+$ recombinants and transductants from a genetically derepressed (*argR*$^-$) strain (514-1).

ArgR$^-$ strains lacking acetylornithinase activity are resistant to acetylnorvaline. Strain 514-1-34 (*argR*$^-$) which is completely deleted for *argE* is resistant. Also, of 52 spontaneous acetylnorvaline-resistant mutants derived from strain 514-1, 45 were *argE*$^-$.

Introduction

N-α-acetyl-L-ornithinase (AO), the enzyme specified by the gene *argE* and under regulatory control of the gene *argR* (Vogel *et al.*, 1971), can deacetylate not only acetylornithine, its natural substrate, but also several other N-acetylated amino acids and amino acid analogs (Vogel and Bonner, 1956; Vogel *et al.*, 1971; Baumberg, 1970). Thus, Baumberg (1970) has shown that acetylhistidine can replace histidine as a growth requirement for histidine auxotrophs with derepressed levels of AO. In these strains addition of arginine to the growth medium inhibits growth due to repression of AO but genetically derepressed (*argR*$^-$) strains are resistant to growth inhibition by arginine. Vogel (unpublished results) obtained similar results using acetylmethionine as a growth requirement for methionine auxotrophs.

Using an N-acetylated derivative of a growth inhibiting amino acid analog, N-α-acetyl-DL-norvaline, which is deacetylated by AO (Baumberg, 1970) we have obtained selective conditions which yield results opposite to those described above, i.e., growth inhibition of *argR*$^-$ strains but not of *argR*$^+$ strains. As described below, this method has been used successfully to select for *argR*$^+$ recombinants.

The results presented below also demonstrate that acetylornithinase activity is required for growth inhibition by acetylnorvaline since the majority of acetylnorvaline-resistant mutants derived from an *argR*$^-$ strain were *argE* mutants. Thus, this method should be useful for generating mutants to study the regulation of the *argECBH* gene cluster.

Materials and Methods

Bacteria. The bacterial strains used are presented in Table 1.

Growth Media. Minimal medium was that of Davis and Mingioli (1950) with glucose (0.5%) or sodium succinate (0.5%) as the carbon source. Solid medium contained 2% agar. When testing for acetylnorvaline resistance 200 μg/ml arginine and an amino acid supplement

Table 1. Description of *E. coli* K12 strains used

Strain	Genotypic and phenotypic characteristics	Source or reference
514	F⁻ *Δlac trp str*	Urm *et al.* (1973)
514-1	F⁻ *Δlac trp str argR*	Urm *et al.* (1973)
514-1-34	F⁻ *Δlac trp str argR rif Δppc-argECBH*	N. Kelker
514-2R-1	F⁻ *Δlac trp str rif Δppc-argECBH*	N. Kelker
AB1927	*Hfr argH metA purF rel sup16 sup48* (see Fig. 2 for direction of transfer)	B. Bachmann

Table 2. Composition of the modified amino acid supplement

Amino acid	Final concentration (μg/ml)
DL-alanine	400
L-aspartic acid	150
L-cysteine	50
L-glutamic acid	200
DL-histidine	75
DL-phenylalanine	100
L-proline	100
L-tyrosine	50
L-threonine	50
L-tryptophan	75

(Table 2) were added. The supplement is a modification of that used by Maas (1972) in that L-leucine, DL-isoleucine, and L-valine have been omitted. Acetylnorvaline was added at 25 mg/ml. For the testing of canavanine sensitivity, minimal medium plus modified amino acid supplement plus 100 μg/ml each of DL-isoleucine, L-leucine, L-valine, L-methionine, L-serine, glycine, L-lysine, and L-canavanine were used.

N-α-Acetyl-DL-Norvaline. This was obtained from Cyclo Chemical Corporation, Los Angeles California. Stock solutions were prepared by mixing 25 g of acetylnorvaline and 40 ml H_2O. Sodium hydroxide (3N) was added slowly with mixing until the acetylnorvaline was completely dissolved and the pH of the solution was 6.9. The final acetylnorvaline concentration was approximately 225 mg/ml. The solution was sterilized by filtration through a millipore filter (0.41 μ pore size) and stored at 4°.

Cell Growth Studies. Strains 514, 514-1, and 514-1-34 were grown for approximately six generations in modified amino acid medium containing arginine and both 0.5% glucose and 0.5% sodium succinate. The cells were centrifuged and resuspended to one-fourth the original volume in unsupplemented minimal medium and 0.2 ml was inoculated into sidearm flasks containing 12 ml of the medium. The cultures were incubated with vigorous rotary shaking in a 37° water bath. Absorbancy measurements were made at 520 mμ using a Coleman Spectrophotometer, Model 6A.

N-α-Acetyl-L-Ornithinase Assays. AO activity was measured by the method of Vogel and Bonner (1956) in sonicates of log phase cells.

Selection for Acetylnorvaline-Resistant Mutants of Strain 514-1. One-tenth ml of an overnight modified amino acid medium plus arginine culture of strain 514-1 was spread on modified amino acid agar plus arginine plus acetylnorvaline. Resistant mutant colonies appeared after 36 hr at 37°. These were purified by isolation of single colonies on

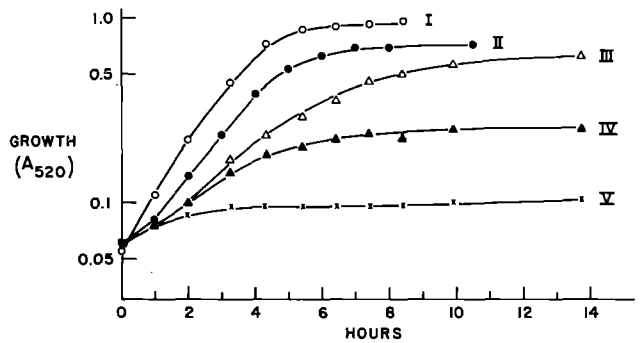

Fig. 1. Growth of 514, 514-1, and 514-1-34 in modified amino acid medium containing 25 mg/ml acetylnorvaline. Curve *I*; strain 514 grown in modified amino acid medium plus arginine. The growth curves of strain 514 grown in the absence of arginine, 514-1 grown in the presence or absence of arginine, and 514-1-34 grown in the presence of arginine are not shown. These were not significantly different from curve I. Curve *II*; strain 514-1-34 grown with arginine and acetylnorvaline. Curve *III*; strain 514 grown with arginine and acetylnorvaline. Curve *IV*; strain 514 grown without arginine but with acetylnorvaline; Curve *V*; strain 514-1 grown with arginine and acetylnorvaline. The growth response of this strain grown without arginine but with acetylnorvaline was identical to curve V

selective medium plates and replicated onto minimal agar supplemented with 100 μg/ml of either acetylornithine, ornithine, citrulline, or arginine.

Mating. Hfr strain AB1927 and F^- strain 514-1 were grown at 37° to an A_{520} of 0.20 in neopeptone broth. The cultures were then mixed in a ratio of 1 Hfr to 10 F^-. The mixture was incubated at 37° with minimal agitation for 90 min. Five ml of the mating mixture (and of AB1927 and 514-1 separately as controls) were centrifuged and resuspended in 5 ml of modified amino acid medium supplemented with arginine and the cultures were incubated overnight which allowed about 3 generations of growth. This was done to obtain repressed levels of AO in $argR^+$ recombinants so that these would have a selective advantage on acetylnorvaline medium. The mating mixture and the controls were then plated at a dilution of 10^{-3} on modified amino acid agar supplemented with arginine and acetylnorvaline. The plates were incubated at 37°. Recombinants, purified by streaking for single colonies on the selective medium, were analyzed for selected and unselected markers by replica plating.

Transduction. The transduction was done as described by Glansdorff (1965). A lysate of the Pl-like phage 363 prepared by growth on $argR^+$ strain 514-2R-1 (derived from strain 514) was used to transduce strain 514-1. As described for the mating, the transduced cells and, as a control, untransduced cells, were grown in modified amino acid medium containing arginine for approximately three generations before plating at a 10^{-3} dilution on selective medium and incubation at 37°. Transductant colonies were picked and purified by isolation of single colonies on selective medium plates and tested for canavanine sensitivity.

Results

Growth of $argR^+$ and $argR^-$ Strains in 25 mg/ml Acetylnorvaline. Growth of $argR^+$ strain 514 is only slightly inhibited by acetylnorvaline (Fig. 1, curve III) while growth of $argR^-$ strain 514-1 is markedly inhibited (curve V). Strain 514-1-34, which is completely deleted for *argE*, is only slightly inhibited by acetylnorvaline (curve II) even though it is $argR^-$. The degree of growth inhibition by acetylnorvaline is dependent upon the *in vivo* level of AO. Thus, strain 514, under conditions of physiological repression of the arginine regulon, *i.e.*, in the presence

Table 3. Growth requirements for acetylnorvaline-resistant mutants of 514-1

Number of strains	No addition	Minimal medium supplemented with			
		acetyl-ornithine	ornithine	citrulline	arginine
37	−	−	+	+	+
8	±	±	+	+	+
7	± or −	± or −	± or −	± or −	+

Strains 514 and 514-1 grew normally on all media and strain MA 220 (*argA*) grew normally on all but the unsupplemented minimal medium.

of arginine (curve III), grows better than under conditions of physiological derepression, *i.e.*, in the absence of arginine (curve IV). Strain 514-1, which is genetically derepressed for the arginine regulon with a high level of AO activity not subject to repression by arginine, is markedly inhibited (curve V).

The specific activities of AO in these strains under conditions of repression and derepression were measured and these correspond to the growth responses to acetylnorvaline. Thus the specific activity of AO in log phase cells of strain 514 growing in minimal medium plus arginine is 5.0 units per mg protein and in medium minus arginine, 14.0 units per mg protein. The AO activity of strain 514-1 growing in the absence of arginine is 65 and in the presence of arginine it is 67.

Analysis of Acetylnorvaline-Resistant Mutants of Strain 514-1. An analysis of acetylnorvaline mutants derived from strain 514-1 (see Materials and Methods) yielded further evidence for the essential role of AO in the inhibition of growth by acetylnorvaline. Fifty two of these strains were replicated onto minimal agar containing either acetylornithine, ornithine, citrulline, or arginine (Table 3). All the strains required arginine for growth. Forty five were *argE* mutants since they could utilize ornithine but not acetylornithine while the remaining 7 grew not at all or only slightly without arginine; this arginine requirement could not be replaced by any precursor.

Selection for $argR^+$ Recombinants and Transductants. $ArgR^+$ Hfr strain AB1927 was mated with $argR^-F^-$ strain 514-1 (see Materials and Methods). Acetylnorvaline-resistant recombinants which appeared within 24 hr of incubation were tested for the selected marker, $argR^+$, by canavanine sensitivity and for the unselected markers, *strA*, *argH*, and *trp* (Fig. 2). Of those which were $argH^+$, and thus could be tested for canavanine sensitivity, 100% were $argR^+$. Further there was 98% linkage of acetylnorvaline resistance with *strA* which is located $1^1/_2$ minutes from *argR* on the *E. coli* chromosome (Taylor and Trotter, 1972). The more distantly located *argH* and *trp* markers showed 15% and 2% linkage, respectively.

It was also possible to transduce strain 514-1 for $argR^+$ by selecting for acetylnorvaline resistance (see Materials and Methods). Of 47 acetylnorvaline-resistant transductants analyzed, all were canavanine sensitive.

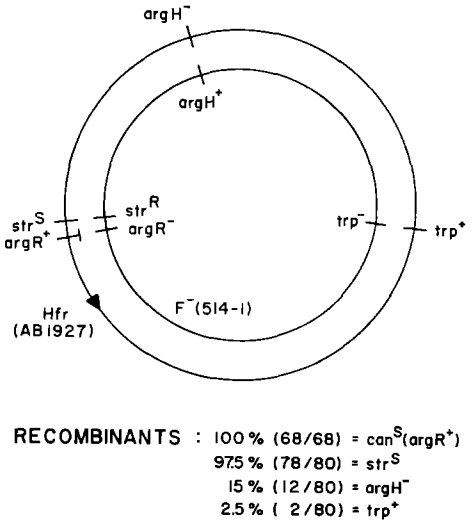

Fig. 2. Conjugation with selection for acetylnorvaline resistance ($argR^+$) between Hrf AB1927 ($argR^+$) and F⁻ 514-1 ($argR^-$)

Discussion

The results presented above clearly show that cells with a lowered level of AO, whether brought about by repression or by mutation to $argE^-$, are resistant to acetylnorvaline whereas those with a high AO level are sensitive.

The large concentration of acetylnorvaline (25 mg/ml) required to inhibit growth of strain 514-1 may be due to the lack of a permease, to a high K_m for deacetylation by AO or to a combination of these factors. In any case, for the purpose of differentiating between $argR^+$ and $argR^-$ strains, it is desirable that acetylnorvaline should not be too efficiently deacetylated. For, if the repressed level of acetylornithinase in $argR^+$ strains were sufficient to generate growth inhibiting levels of norvaline, no distinction between $argR^+$ and $argR^-$ strains would be possible.

Although, as expected, a large number of *argE* mutants was found among acetylnorvaline resistant mutants of 514-1, no $argR^+$ revertants were found. The reason for this is not known but it may be due to the nature of the $argR^-$ mutation in 514-1 since this mutation may not be revertible.

The method reported here should be useful for selection of a variety of mutants which will be helpful in studies of arginine regulation, since any mutation, whether in a regulatory or structural gene, which limits expression of *argE*, confers resistance to acetylnorvaline.

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References

Baumberg, S.: Acetylhistidine as a substrate for acetylornithinase: A new system for the selection of arginine regulation mutants in *Escherichia coli*. Molec. gen. Genet. **106**, 162–173 (1970)

Davis, B. D., Mingioli, E. S.: Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bact. **60**, 17–28 (1950)

Glansdorff, N.: Topography of cotransducible arginine mutations in *Escherichia coli* K-12. Genetics **51**, 167–179 (1965)

Maas, W. K.: Mapping of genes involved in the synthesis of spermidine in *Escherichia coli*. Molec. gen. Genet. **119**, 1–9 (1972)

Taylor, A. L., Trotter, C. D.: Linkage map of *Escherichia coli* strain K12. Bact. Rev. **36**, 504–524 (1972)

Urm, E., Yang, H., Zubay, G., Kelker, N., Maas, W.: *In vitro* repression of N-α-acetyl-L-ornithinase in *Escherichia coli*. Molec. gen. Genet. **121**, 1–7 (1973)

Vogel, H. J., Bonner, D. M.: Acetyl ornithinase of *Escherichia coli*: Partial purification and some properties. J. biol. Chem. **218**, 97–106 (1956)

Vogel, R. H., McLellan, W. L., Hirvonen, A. P., Vogel, H. J.: The arginine biosynthetic system and its regulation. In: Metabolic regulation, vol. V, p. 463–488, ed. by H. Vogel. New York: Academic Press 1971

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