***Bacillus subtilis* 168 Mutants Resistant to Arginine Hydroxamate in the Presence of Ornithine or Citrulline**

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(Received 10 October 1983; revised 17 January 1984)

Mutations in *Bacillus subtilis* 168 have been isolated that confer resistance to arginine hydroxamate in the presence, but not absence, of ornithine. Seven such Ahr mutants have been studied in detail. In common with certain classes of Ahr mutant (resistant to arginine hydroxamate in the absence of arginine precursors) described previously, these Ahr mutants showed little or no inducibility of enzymes of arginine catabolism. Mutants that showed no inducibility were unable to utilize arginine or ornithine as sole nitrogen source. The only biosynthetic enzyme to show any consistent differences in activity from the parent was ornithine carbamoyltransferase, whose level was slightly elevated in cells grown in the presence of ornithine or citrulline. PBS1 transduction crosses showed that two of the *ahor* mutations map at the *ahrA* locus, while a third (unique in its resistance to arginine hydroxamate in the presence of citrulline) mapped at a hitherto undescribed locus closely linked to *metC*, designated *ahrD*.

INTRODUCTION

Details of molecular mechanisms for regulation of expression of bacterial genes encoding metabolic enzymes are as yet available only for *Escherichia coli* and *Salmonella typhimurium*. In the hope of determining whether unfamiliar control devices might be found in an unrelated group, we have been studying as a model system regulation in the pathways of arginine biosynthesis and catabolism in *Bacillus subtilis* 168. In this organism, the three readily assayable biosynthetic enzymes *N*-acetylornithine δ -aminotransferase (AcOAT), ornithine carbamoyltransferase (OCT) and argininosuccinase (AS) are repressed in the presence of arginine; the catabolic enzymes arginase (A) and ornithine δ -aminotransferase (OAT) are induced in the presence of arginine or proline, this induction being antagonized (presumably via some form of nitrogen metabolite repression) more effectively by glutamine than ammonia (Harwood & Baumberg, 1977; Baumberg & Harwood, 1979). Harwood & Baumberg (1977) showed that mutants resistant to the arginine analogue arginine hydroxamate (AH) often showed alterations in expression of these enzymes, usually involving loss or non-inducibility of these two catabolic enzymes but sometimes also simultaneous loss of repressibility of the biosynthetic ones. Mountain & Baumberg (1980) reported that mutations conferring resistance to AH mapped at three unlinked loci on the *B. subtilis* chromosome, *ahrA*, *B* and *C*; mutations resulting only in altered expression of the catabolic enzymes mapped at *ahrA* and *B*, while the two mutations resulting in simultaneous loss of inducibility of the catabolic enzymes and of repressibility of the biosynthetic ones mapped at *ahrC*.

Abbreviations: AcOAT, *N*-acetylornithine δ -aminotransferase; OCT, ornithine carbamoyltransferase; AS, argininosuccinase; A, arginase; OAT, ornithine δ -aminotransferase; AH, arginine hydroxamate; Ahr/s, resistant/sensitive to arginine hydroxamate; Ahr/s, resistant/sensitive to arginine hydroxamate in the presence of ornithine; Aut^{+/-}, able/unable to utilize arginine as sole nitrogen source; Out^{+/-}, able/unable to utilize ornithine as sole nitrogen source; *ahr-x*, mutation conferring an Ahr phenotype and not assigned to a particular locus; *ahor-x*, mutation conferring an Ahr phenotype and not assigned to a particular locus; *ahrX*, a locus of *ahr-x* or *ahor-x* mutations.

We describe here the properties of mutants selected as resistant to AH in the presence of the arginine precursors ornithine or citrulline, but sensitive to it in their absence. The rationale for this procedure was that the enzyme phenotypes of AH-resistant isolates suggested that selection was effectively for intracellular accumulation of arginine (in these cases through deficiency in its breakdown), this then in some way antagonizing the inhibitory effect of AH. It was hoped that the provision of large intracellular levels of ornithine or arginine might, in hypothetical mutants with elevated levels of enzymes mediating the conversion of these to arginine, produce high endogenous levels of arginine and thereby lead to AH resistance: such mutants might include ones with the hitherto undiscovered phenotype of elevation of levels of biosynthetic enzymes without change in the catabolic ones.

METHODS

Bacterial strains and media. The bacterial strains used are listed in Table 1. Methods of maintenance, nutrient and minimal media and supplements were as in Mountain & Baumberg (1980). AH was used at $100 \mu\text{g ml}^{-1}$; ornithine and citrulline in the presence of AH were at $1000 \mu\text{g ml}^{-1}$; and arginine and ornithine as sole nitrogen source were present at $300 \mu\text{g ml}^{-1}$.

Growth, harvesting of cells, permeabilization of cells by freezing and thawing, and enzyme and protein assays. These were carried out as described by Mountain & Baumberg (1980), except that AcOAT was assayed by the method of Albrecht & Vogel (1964).

PBS1-mediated transduction. This was carried out according to Mountain & Baumberg (1980).

Table 1. *Strains of B. subtilis*

Strain no.	Genotype	Source/Reference
EMG50	Prototroph	Laboratory collection
BC369	<i>argC4 metD1 hisA1 aut-1</i>	Young <i>et al.</i> (1969); Mountain & Baumberg (1980)
UL300	<i>argC4 metD1 hisA1</i>	A. Mountain; Aut ⁺ transformant of BC369
AM111	<i>aut-1</i>	Mountain & Baumberg (1980)
Ahr2-52	<i>ahrA52</i>	Mountain & Baumberg (1980)
QB123	<i>trpC2 sacA321 ctrA1</i>	Dedonder <i>et al.</i> (1977)
QB934	<i>trpC2 aroD120 lys-1</i>	Dedonder <i>et al.</i> (1977)
QB944	<i>trpC2 purA16 cysA14</i>	Dedonder <i>et al.</i> (1977)
MB21	<i>metC leuA</i>	P. Piggot

RESULTS AND DISCUSSION

Isolation and properties of Ahr mutants

Cells of the prototrophic sub-strain EMG50 of *B. subtilis* 168, suspended in minimal salts-glucose medium, were plated on minimal salts-glucose agar containing AH together with either ornithine or citrulline. After 24 h, colonies had developed on both media; after a further 24 h, additional small colonies, clearly distinguishable from those appearing earlier, had appeared on AH + ornithine but not on AH + citrulline. 217, 69 and 407, respectively, of these isolates were patched on the appropriate selecting medium and replicated to minimal salts-glucose plates containing, with ammonia as nitrogen source: AH; AH + ornithine; AH + citrulline; and without ammonia: arginine or ornithine as nitrogen source. It was found that all the isolates that developed rapidly on AH + ornithine or citrulline grew on the other selective medium and also, more importantly, on AH alone. Also, 36% of those selected on AH + citrulline and 65% of those selected on AH + ornithine had partially or completely lost the capacity to use arginine and/or ornithine as sole nitrogen source (i.e. were Aut⁻ and/or Out⁻). This latter phenotype was common among the isolates selected on AH alone described by Harwood & Baumberg (1977) and Mountain & Baumberg (1980). On the other hand, only 12% of the isolates that developed slowly on AH + ornithine grew on AH alone – these, but not the rest, also grew on AH + citrulline – though about 75% showed some deficiency in arginine or ornithine utilization.

These results suggested that the rapidly developing isolates were unlikely to differ significantly from the previously characterized Ahr mutants. The isolates that developed slowly

Table 2. Growth of *Ahor* mutants on AH with and without ornithine or citrulline and on arginine or related amino acids as sole nitrogen source

Growth was tested on minimal salts-glucose agar supplemented as shown. Plates were scored after 24 h at 37 °C.

	Growth* on:			
	AH	AH + ornithine	AH + citrulline	Arginine, ornithine or citrulline as sole N source
EMG50	—	—	—	++
Ahr2-52	++	++	++	—
AM111	—	+	—	—
Ahor1	—	+	—	+†
Ahor7	—	++	++	+++‡
Ahor10	—	++	—	±
Ahor13	—	+	—	+++‡
Ahor17, 23, 24	—	+	—	—

* ++, Good growth; +, moderate growth; ±, weak growth; —, no growth.

† No growth on citrulline.

‡ Moderate growth on citrulline.

on AH + ornithine and could not grow on AH alone represented a new phenotype (termed *Ahor*), and further work was restricted to them. The fact that most of them were to some extent *Aut*[−] and/or *Out*[−] indicated that these at least, contrary to our hopes, would probably turn out to be affected in expression of catabolic enzymes. It may also be noted that although the *Ahor* isolates grew from single cells to colonies in their original selection only in 2 d, on subsequent testing they grew on the same medium in 1 d; this would be consistent with the occurrence of secondary mutations in at least some cases. Seven non-sibling isolates, designated *Ahor*1, 7, 10, 13, 17, 23 and 24, were chosen for more detailed investigation.

Growth properties of Ahor mutants

Plate tests were carried out to determine the growth characteristics of the selected *Ahor* strains on media containing AH alone or combined in various concentrations with ornithine or citrulline, or including various nitrogen sources in place of ammonia. These tests were carried out simultaneously on (1) the parental EMG50, (2) the *Ahr* strain *Ahr*2-52, an example of Class 1 of Harwood & Baumberg (1977) whose loss of expression of the catabolic enzymes is most extreme, and (3) AM111, a prototrophic derivative of strain BC369, which had been found earlier to be phenotypically *Ahor* and also *Aut*[−]/*Out*[−] (Mountain & Baumberg, 1980). The results are given in Table 2, and can be summed up as follows. AM111 and the new *Ahor* isolates, with the exception of *Ahor*7, are unable to grow on AH, as already indicated, or AH + citrulline (their capacity to grow on AH + ornithine is also diminished as the AH concentration rises or the ornithine concentration drops; data not shown). *Ahor*7, in sharp contrast, grows well on AH + citrulline and on the various combinations of AH and ornithine concentrations tested. As regards use of arginine, ornithine, or citrulline as nitrogen source, there is a gradation among the *Ahor* isolates from AM111 and *Ahor* mutants 17, 24 and 24, which are totally unable to utilize any of these, through *Ahor*10 to *Ahor*1 and finally *Ahor*7 and *Ahor*13. As expected, all strains tested grow well with proline as sole nitrogen source (data not shown).

Enzyme activities in Ahor isolates

Assays for A, OAT, AcOAT, OCT, AS and protein were performed on permeabilized suspensions of EMG50, AM111 and the seven new *Ahor* isolates. Specific activities are shown in Table 3 (those for *Ahor*17 and 23 are omitted since these isolates behaved like *Ahor*24). It is seen that uninduced levels of A (i.e. in cells grown in unsupplemented medium) in the *Ahor* isolates are either similar to those in EMG50 or less than a factor of two lower; in the presence of arginine, *Ahor*1, 10, and 24 show no induction of this enzyme while *Ahor*7 and 13 show a

Table 3. *Specific activities of arginine enzymes in Ahor mutants*

Cultures were grown at 37 °C to mid-exponential growth phase in minimal salts-ammonia-glucose medium supplemented as indicated, with chloramphenicol added to 100 µg ml⁻¹. Cells were harvested, frozen and thawed cell lysates prepared and enzyme and protein assays performed as referred to in Methods.

Growth supplement	...	Specific activity*																			
		None				Arginine				Ornithine				Citrulline							
		A	OAT	AcOAT	OCT	AS	A	OAT	AcOAT	OCT	AS	A	OAT	AcOAT	OCT	AS					
Strain	Enzyme	4.9	0.4	0.3	4.0	0.15	114	5.2	u	0.3	u	110	6.8	0.4	14	0.6	92	7.0	u	2.5	0.2
EMG50		2.9	u	0.1	4.2	u	3.1	u	u	0.3	0.1	4.0	0.0	0.1	14	0.35	3.5	u	u	4.3	0.1
AM111		7.0	0.15	0.4	6.4	0.1	5.1	0.1	u	u	0.05	4.9	0.1	0.4	18	0.4	5.4	0.35	u	7.4	0.1
Ahor1		5.9	0.1	0.4	7.0	0.1	19	0.4	u	0.8	0.05	13	0.25	0.4	12	0.3	12	0.8	u	8.1	0.15
Ahor7		5.7	0.15	0.4	5.6	0.15	6.4	0.1	u	u	0.05	6.5	0.15	0.3	17	0.35	5.8	0.25	0.2	16	0.15
Ahor10		6.4	0.15	0.3	4.3	0.05	16.5	0.45	u	u	0.1	14	0.45	0.4	>20	0.3	8.7	0.75	u	6.0	0.1
Ahor13		3.0	0.1	0.3	6.0	0.15	3.5	0.15	u	0.3	0.05	4.1	0.1	0.4	17	0.3	4.6	0.35	0.1	7.9	0.1
Ahor24																					

* Specific activities are given in µmol product (mg protein)⁻¹ h⁻¹ for A, OCT and AS, and in A₄₄₀ (mg protein)⁻¹ h⁻¹ for OAT and AcOAT. u, Unmeasurably small.

roughly threefold induction, with ornithine and citrulline giving similar results except that the induction is slightly less. OAT behaves similarly to A, though induction by citrulline in all the isolates is two to three times that by arginine and ornithine. These results accord with the superior ability of Ahor7 and 13 in utilizing these amino acids as sole nitrogen source. These figures are similar to those for Ahr mutant classes 3 and 6 in which there is no, or barely, detectable induction of these enzymes (Mountain & Baumberg, 1980).

As regards the biosynthetic enzymes, the only clear-cut difference from EMG50 is the elevated level of OCT in citrulline-grown cells of all Ahor strains by comparison with EMG50, by a factor of about two to over six. Possibly significant are the increased levels of OCT (again in comparison to EMG50) in most of the isolates in unsupplemented medium (by, however, a factor of less than two) and in Ahor7 and 17 in medium supplemented with arginine. These OCT levels are reminiscent of those in the sole representative of class 5, Ahr1-120 (Mountain & Baumberg, 1980), though this strain is less affected in expression of the catabolic enzymes than any described here, induction being about half that found with EMG50. It is clear, in any case, that the enzyme activity characteristics of the Ahor isolates differ at least slightly from those of any of the Ahr isolates previously described.

The AH resistance phenotype of these isolates is therefore roughly in accord with the generalization that this resistance is greater the lower the levels of the catabolic enzymes under inducing conditions. Comparing the data with those of Mountain & Baumberg (1980), we see that apart from their Class 5 mutant Ahr1-120 (which in their hands seemed to have reverted to AH sensitivity) the only exception to this generalization is the Class 4 mutant Ahr2-48, in which inducibility of A and OAT is under all conditions superior to that of the Ahor strains, and yet which is resistant to AH in the absence of ornithine. Within the Ahor isolates, there is good correlation between inducible levels of the catabolic enzymes and ability to utilize arginine or its precursors as nitrogen source: the isolates with the highest residual inducibility, Ahor7 and 13, can do so, the others cannot. However, if we include the Ahr strains in the comparison, matters become more complex in that the levels of A and OAT in Ahor17, 23 and 24, which do not utilize arginine or its precursors, are similar to those in members of Class 3 of Mountain & Baumberg (such as Ahr2-34) which do. Finally, the enzyme activities give no clue to the unique phenotype of Ahor7 in being able to grow on AH + citrulline.

Mapping of ahor mutations

To locate the *ahor* mutations responsible for the Ahor phenotype of these isolates, linkage was first sought in PBS1-mediated transduction with markers found by Mountain & Baumberg (1980) to be linked to *ahrA*, *B* and *C*. Accordingly, PBS1 lysates made on each Ahor strain were used to transduce recipients QB123 (*sacA* linked to *ahrA* and *ahrB*, *ctrA* to *ahrB*); QB934 (*aroD* and *lysA* both linked to *ahrC*); QB944 (*cysA* and *purA* both linked to *ahrA*); and UL300, this being included since the only biosynthetic enzyme affected in the Ahor set is the OCT encoded by *argC*, this gene being linked to *metD*.

No linkage was found of any *ahor* marker with *sacA*, *ctrA*, *aroD* or *lysA*. Also, mutations *ahor-1*, *-10*, *-13* and *-24*, showed no linkage with any marker tested. It should be noted, however, that in several cases particular combinations of lysate and recipient gave very few transductants so that weak linkage might have been missed. Results in cases where linkage was detected are given in Table 4. It is seen that *ahor-17* and *-23* show the linkage with *cysA* and *purA* characteristic of *ahrA* mutations; when *Pur*⁺, but not *Cys*⁺, transductants were scored separately for the Ahor/s and Out^{+/-} properties, combinations of these different from either parent were observed yielding the complicated picture described by Mountain & Baumberg (1980). *ahor-7* was found to be cotransducible with *metD1* and *argC4*, the distribution of unselected markers indicating the order *metD-argC-ahor-7* which is the only one yielding Ahos Met⁻ and Ahos Arg⁻ as the rarest recombinant classes among transductants selected as Arg⁺ and Met⁺, respectively. *ahr-7* therefore appeared to map at a new *ahr* locus which we name *ahrD*, on the side of *argC* opposite to *metD*. A suitable marker in this region with which to test linkage is *metC*. Accordingly, PBS1 grown on Ahor7 was used to transduce MB21 to Met⁺. As indicated in Table 4, strong linkage (about 95%) was found. We have not attempted to map

Table 4. *Results of transductional crosses with Ahor donors*

Recipient	Selection for:	Donor	Frequency*		
			Ahor7	Ahor17	Ahor23
QB944	CysA ⁺		0/34	8/52	2/25
	PurA ⁺		0/46	46/55	29/37
UL300†	ArgC ⁺		33/119‡	0/111	0/111
	MetD ⁺		11/148‡	NT	NT
MB21	MetC ⁺		68/71	NT	NT

* NT, Not tested.

† The Ahor/s character was scored in transductants of this recipient with AH at 75 µg ml⁻¹.

‡ The frequencies of the various unselected marker combinations in these transductants were as follows. Selection for ArgC⁺: Ahor MetD⁺ 20; Ahor MetD⁻ 13; Ahos MetD⁺ 84; Ahos MetD⁻ 2. Selection for MetD⁺: Ahor ArgC⁺ 11; Ahor ArgC⁻ 0; Ahos ArgC⁺ 118; Ahos ArgC⁻ 19.

ahrD7 any further; however, it is tempting to speculate whether it might show any connection with the *pro(AB)* locus reported by Harwood (1974) and Buxton (1980) to be strongly linked to *metC*. The phenotype of *pro(AB)* mutants, a requirement for arginine or proline, indicates a block in the proline biosynthetic pathway between glutamate and glutamic-γ-semialdehyde; this pathway operates in the reverse direction in the catabolism of arginine or proline to glutamate. This locus also appears to be the site of mutations conferring resistance to the proline analogue L-azetidine-2-carboxylate (Gallori *et al.*, 1978), which may be regulatory in nature.

We thank R. Dedonder, J.-A. Lepesant and P. Piggot for strains. J. McChesney assisted in some of the mapping. This work was supported by MRC grant G974/223/B to S.B.

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