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Map Locations of Some Mutations Conferring Resistance to Arginine Hydroxamate in *Bacillus subtilis* 168

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Summary. Mutations conferring resistance to arginine hydroxamate in *Bacillus subtilis* 168 have been located on the genetic map by PBSl-mediated transduction. The majority of these mutations, belonging to classes 1, 2 and 4 of Harwood and Baumberg (1977) and affecting only expression of arginine catabolic enzymes, map at a locus designated *ahrA* cotransducible with *cysA*, *purA* and *sacA*. The order of markers in this region appears to be *sacA–ahrA–purA–cysA*. Certain anomalies were observed in the properties of Pur^+ transductants from crosses with an Ahr donor and a *purA* recipient. A single *ahr* mutation (class 3), also affecting only arginine catabolism, maps between *ctrA* and *sacA* at a locus designated *ahrB*. Two others (class 6), affecting simultaneously enzymes of both arginine biosynthesis and catabolism, map between *lys* and *aroD* at a locus designated *ahrC*. Preliminary attempts to define the nature of functional products specified by these *ahr* loci suggest that a protein is encoded at *ahrA*.

Introduction

Remarkably little is know about the molecular mechanisms regulating gene expression in prokaryotes outside the enterobacteria. It is true that phenomena such as induction of catabolic enzymes and repression of anabolic ones are common among most classes of prokaryotic (and for that matter eukaryotic) microorganisms. However, it cannot be assumed that in non-enterobacterial organisms these physiological control devices are the result of molecular events similar to those known to occur in *Escherichia coli* and *Salmonella typhimurium*, such as: binding of repressor to operator blocking access of RNA polymerase to a promoter, facilitation of RNA polymerase attachment to a promoter by binding of a regulatory protein at an adjacent DNA site, and modulated termination of transcription (with the regulatory proteins involved in any of these often existing in ligand-influenced equilibrium between forms differing in activity). It is likely that similar mechanisms apply in at least some other bacteria, e.g. *Pseudomonas aeruginosa* (Farin and Clarke, 1978, and references therein), though they may be combined in unfamiliar ways. The possibility of further novel combinations or new kinds of molecular mechanism might be usefully pursued in model species as unlike *E. coli* as possible. *Bacillus subtilis* suggests itself as a suitable organism, in being Gram-positive and relatively amenable to genetic analysis. We have chosen to study, from the viewpoint of the genetics of regulation, the pathways of arginine metabolism, the physiology of which has been examined to some extent in this organism (Harwood and Baumberg, 1977; Baumberg and Harwood, 1979) as well as in the related *Bacillus licheniformis* (Broman et al., 1978). This system has the attraction of resembling biochemically its well-researched equivalent in the eukaryotic microorganism *Saccharomyces cerevisiae* (Dubois et al., 1978; Messenguy, 1979; and references therein).

It has been shown (Harwood and Baumberg, 1977) that Ahr mutants of *B. subtilis*, i.e. resistant to the analogue arginine hydroxamate, carry pleiotropic mutations – possibly regulatory in nature

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Abbreviations. OAT, ornithine aminotransferase. OCT, ornithine carbamoyltransferase; AS, argininosuccinase; AH, arginine hydroxamate; Ahs, Ahr, arginine hydroxamate-sensitive or -resistant; Ahos, Ahor, sensitive or resistant to arginine hydroxamate in the presence of ornithine; *ahr*, allele conferring arginine hydroxamate resistance; Out^+, Out^-, able or unable to utilise ornithine as sole nitrogen source

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– affecting production of the arginine catabolic enzymes arginase and OAT. In the wild-type, these enzymes are induced in the presence of arginine or its precursors ornithine and citrulline. In Ahr mutants, levels of the catabolic enzymes are low during growth under conditions that permit induction in the wild-type. The phenotypes are varied, however, and six classes were distinguished as to: (1) extent of residual inducibility; (2) whether the enzymes were equally affected; (3) which metabolites failed to cause induction, and (4) involvement of arginine biosynthetic enzymes as well – in members of class 6 one biosynthetic enzyme (OCT), and in class 5 another also (AS), were reported to be present at elevated levels in comparison to wild-type under at least some growth conditions.

Preliminary mapping data were also presented in the paper cited. It was reported that among single representatives of each mutant class, members of classes 1–5 carry *ahr* mutations linked by PBS1 transduction to *cysA14;* the member of class 6, however, did not. In this paper we describe further transduction experiments which confirm the existence of an *ahr* locus linked to *cysA14,* and reveal two others located respectively between *ctrA1* and *sacA321,* and *aroD120* and *lys-1/-21.* The loci have been designated *ahrA, B* and *C* respectively.

Materials and Methods

Bacterial and Phage Strains and Media. The strains used are listed in Table 1. Bacteria were stored for periods of up to six months on nutrient agar plates and for longer periods in nutrient broth containing glycerol (10% v/v) at −20° C. The poorly viable strain HA101B carrying *sup-1* was maintained by restreaking fortnightly on selective medium (minimal salts-glucose + leucine). Nutrient media, minimal media and supplements were as described in Harwood and Baumberg (1977).

Growth and Harvesting of Bacteria. Where bacteria were grown for the preparation of lysates for enzyme assay, single colonies from nutrient agar plates not more than one month old were inoculated into 4 ml appropriately-supplemented minimal medium in 150 × 19 mm test tubes, the cultures being incubated overnight at 37° C with vigorous shaking. 1 ml overnight culture was diluted into 50 ml of the same medium in a 250 ml side-arm flask which was then incubated at 37° C with shaking. Growth was followed turbidimetrically on a Klett-Summerson colorimeter (no. 66 filter). At 50–80 units, the culture was again diluted 50-fold into the same medium and incubation continued under identical conditions. On once more reaching 50–80 units, cultures were harvested and cells rendered permeable for enzyme and protein assays by freezing and thawing as described by Harwood and Baumberg (1977).

Enzyme Assays. Arginase, ornithine aminotransferase, argininosuccinase and protein were assayed as described in Harwood and Baumberg (1977). The ornithine carbamoyltransferase assay was performed also as described by these authors except with the substitution of 12 μmoles EDTA, pH 8.75 for Tris-HCl, pH 8.4 (see also Issaly and Issaly, 1974). Enzyme activities as given in Tables 2 and 10 below refer to representative individual experiments and are not means of any kind from different runs. The number of experiments (each involving strains being cultured in each growth medium mentioned and being assayed for each enzyme) was three for Table 2 and two for Table 10.

Transduction with Phage PBS1 was performed essentially as described by Young and Wilson (1974) except that new stocks were prepared every three months and titred on strain EMG50 in 0.3% nutrient agar overlays, which gave reasonably sized plaques. Donor lysates were routinely screened for transducing activity using a recipient carrying two markers known to be readily cotransducible – usually *dal-1* and *narB1* in strain QB817, with selection for Dal^+ transductants because these can be picked after 24 h incubation at 37° C on nutrient medium lacking D-alanine. *dal-1* and *narB1* are usually c. 30% cotransducible. Transductants for most markers were visible after 24 h, were picked and purified after 42 h, and were then screened for unselected markers by replica plating. Sac^+/Sac^- was tested on minimal salts with 0.1% sucrose; both Nar^+/Nar^- and Ctr^+/Ctr^- on minimal salts-glucose with 0.2% KNO_3 as sole nitrogen source; and Ahr/Ahs on minimal salts-glucose with AH (200 μg/ml unless otherwise stated). The Out^+/Out^- phenotypes (ability/inability to utilise ornithine as sole nitrogen source) were somewhat difficult to score and replica plating could not be employed. Instead these phenotypes were tested by restreaking transductants on to plates with this compound (300 μg/ml as the monohydrochloride) as nitrogen source, concentrations of all other potential nitrogen sources added to satisfy auxotrophic requirements (e.g histidine) being minimised. Suitable Out^+/Out^- controls were always included on each plate. Rifampicin-resistant (Rif^r) transductants were selected as in Harford and Sueoka (1970). The *rif-2* allele appears to be dominant to rif^+, so that transduction mixtures can be plated on selective media without allowing for segregation (P. Piggot, personal communication).

Sensitivity to Phages. Transductants were spot-tested for sensitivity to phages SP01 and SP01 *Fsus51* when streaked 12 to a plate on 1% CY medium (Okubo and Yanagida, 1968).

Chemicals were as described by Harwood and Baumberg (1977).

Results

1. Phenotypes of Mutants Employed in this Study

The single representatives of each Ahr class used by Harwood and Baumberg (1977) in preliminary PBS1 transductional mapping experiments were also chosen for investigation here. They were: class 1, Ahr 2–52; class 2, Ahr 2–8; class 3, Ahr 2–34; class 4, Ahr 2–48; class 5, Ahr 1–120; and class 6, Ahr 2–14. A further representative of class 6, Ahr 2–9, was also included. Another strain of interest was BC369; preliminary experiments by Harwood and Baumberg (1977) suggested that it harbours a mutation (to be termed here *aut-1*) which may be in the structural gene for OAT, while they favoured the view that the other Ahr isolates carry regulatory mutations of various kinds. A prototrophic derivative (Met^+ His^+ Arg^+) of BC369, strain AM111, was constructed by PBS1 transduction and was also investigated here.

Table 1. Bacterial and phage strains

Strain	Marker(s) used in locating the *ahr* mutations	Remainder of genotype	Source/Reference
(a) Bacteria[a]			
EMG50		Prototroph	Harwood and Baumberg, 1977
Ahr 2–52		*ahr2–52*	
Ahr 2–8		*ahr2–8*	
Ahr 2–34		*ahr2–34*	
Ahr 2–48		*ahr2–48*	
Ahr 1–120		*ahr1–120*	
Ahr 2–9		*ahr2–9*	
Ahr 2–14		*ahr2–14*	
Ahr 2–52 Rif^r		*ahr2–52 rif*	Spontaneous Rif^r derivative of Ahr 2–52
Ahr 2–8 Rif^r		*ahr2–8 rif*	Spontaneous Rif^r derivative of Ahr 2–8
BC369		*argC4 metD1 hisA1 aut-1*	Young et al., 1969
AM111		*aut-1*	Arg$^+$ Met$^+$ His$^+$ transductant of BC369
BD111	*thr-5, cysB3*	*trpC2*	P. Piggot
AM830		*thr-5 hisB5 cysB3*	Trp$^+$ *hisB5* transductant of BD111
BD112	*cysA14*		Harwood and Baumberg, 1977
CU173	*citK1*	*trpC2 ilvA1*	J.A. Lepesant/Harford, 1975
GSY292	*trpC2, gltA292*		Dedonder et al., 1977
GSY1020	*purB6, hisB2*		Piggot and Taylor, 1977
HA101B		*hisB5 metB5 leuA8 sup-1*	D. Shub/Tevethia et al., 1974
MB21	*metC3*	*leuA8 tal-1*	Piggot and Taylor, 1977
MB26	*leuA8, pheA12*	*rif-2*	P. Piggot
MB178	*argA3*	*lys-21 metB5 pheA12*	Harwood and Baumberg, 1977
AM210		*lys-21 metB5 pheA12*	Arg$^+$ transductant of MB178
MB181	*argC4, hisA1, ura-1*		Harwood and Baumberg, 1977
QB123		*trpC2 sacA321 ctrA1*	Dedonder et al., 1977
QB687	*sacA321, tre-12*	*trpC2*	Lepesant-Kejzlarová et al., 1975
QB698	*ald-1*	*trpC2 sacQ36*	
QB817	*narB1, dal-1*	*metB5 sacA321*	
QB935		*trpC2 aroD120 lys-1*	Dedonder et al., 1977
QB944		*trpC2 purA16 cysA14*	
SB120		*trpC2 aroD120*	J.-A. Lepesant/Harford, 1975
SB202		*trpC2 aroB1*	J.-A. Lepesant
VB151		*leuA8 metB5 lysS1*	P. Piggot/Racine and Steinberg, 1974
168TT	*thyA1, thyB1*	*trpC2*	Lepesant-Kejzlarová et al., 1975
(b) Phages			
PBS1		Wild type	P. Piggot
SPO1		Wild type	J N. Reeve
SPO1–F51		*Fsus51*	D. Shub

[a] All strains are derivatives of *B. subtilis* 168

Specific activities of two catabolic enzymes (arginase and OAT) and two biosynthetic ones (OCT and AS) were measured in frozen and thawed lysates of these strains and of the Ahs parent EMG50 after growth in minimal salts-glucose media without an amino acid supplement or with arginine, ornithine or citrulline supplements (Table 2). The results of plate tests on these strains determining their ability to grow on arginine hydroxamate in the presence and absence of ornithine and to utilise arginine or ornithine as sole nitrogen source are shown in Table 3.

The results in Table 2 for the Ahs parent EMG50 show general agreement with those of Harwood and Baumberg (1977). The arginine catabolic enzymes arginase and OAT are induced by arginine, ornithine and citrulline, but the biosynthetic ones OCT and AS are repressed by arginine only. Specific activities of both biosynthetic enzymes are somewhat higher than in Harwood and Baumberg's experiments – in the case of OCT no doubt as a result of substituting EDTA for Tris in the assay mixture, Tris having been found to be inhibitory (V. Stalon, personal communication). Although Harwood and Baumberg found AS activity undetectable except in some Ahr mutants, we now find levels of this enzyme to be low but measurable when assays are performed immediately after preparing the frozen and thawed lysates. A reproducible two- to three-fold increase in both OCT

Table 2. Specific activities of arginine enzymes in parent and Ahr mutants

Strain	Genotype	Growth supplement															
		None				Arginine (1000 µg/ml)				Citrulline (1000 µg/ml)				Ornithine (1000 µg/ml)			
		Enzyme.				Enzyme:				Enzyme.				Enzyme.			
		A[a]	OAT	OCT	AS	A	OAT	OCT	AS	A	OAT	OCT	AS	A	OAT	OCT	AS
EMG50	Prototroph wild-type	4.9	0.4	4.0	0.15	114	5.2	0.3	u[b]	92	7.0	2.5	0.2	110	6.8	13.7	0.6
Ahr 2–52 (class 1)[c]	Prototroph *ahr2–52*	u	u	1.7	0.1	u	u	0.2	u	u	0.2	1.5	0.1	u	0 5	9.0	0.1
Ahr 2–8 (class 2)	Prototroph *ahr2–8*	u	u	2.0	0.2	u	u	0.3	u	u	1.0	2.3	0.1	u	0.1	10.1	0.2
Ahr 2–34 (class 3)	Prototroph *ahr2–34*	3.0	0.1	3.1	0.1	2.0	0.2	0.3	u	3.0	0.2	3.0	0.1	2.0	0.2	8.6	0.1
Ahr 2–48 (class 4)	Prototroph *ahr2–48*	3.0	0 3	3.0	u	85	1.2	1.0	u	100	3.8	1.5	0.1	24	1.0	8.0	0.1
Ahr 1–120 (class 5)	Prototroph *ahr1–120*	2.0	0.2	8 0	0.2	55	2 5	0.5	u	95	4.0	5.0	0 3	39	2.0	12.0	0.5
Ahr 2–9 (class 6)	Prototroph *ahr2–9*	5.0	0.4	>15	1.5	3.1	1.0	12.2	0.2	2.0	0.7	7.5	0.3	4.3	0.3	>15	2 2
Ahr 2–14 (class 6)	Prototroph *ahr2–14*	4.1	0.2	12.1	0.3	2.4	0.5	6.1	u	2.8	0.6	13.2	0.2	4.0	0.6	12.8	0.6
BC369	*argC4 metD1 hisA1 aut-1*	2.0	u	u	0 02	2.7	u	u	u	3 4	u	u	0.1	4.3	u	u	0.2
AM111	Prototroph *aut-1*	2.9	u	4.2	0.01	3.1	u	0.3	0.01	3.5	u	4.3	0.1	4.0	u	13.7	0.3

Cultures were grown at 37° C to mid-exponential growth phase in the media indicated, chloramphenicol added to 100 µg/ml and cells harvested by centrifugation. Frozen and thawed cell lysates were prepared and enzyme and protein assays performed as described in Materials and Methods. Specific activities are given in µmoles product (h) mg protein for A, OCT and AS, in A_{440}(h) mg protein for OAT

[a] A = arginase
[b] u = unmeasurably small
[c] According to the classification of Harwood and Baumberg (1977)

Table 3. Growth of the parent strain and Ahr mutants on arginine hydroxamate with and without ornithine and on arginine or ornithine as sole nitrogen source

	AH (100 µg/ml)		AH (200 µg/ml)		AH (100 µg/ml) + ornithine (1000 µg/ml)		Arginine or ornithine (300 µg/ml) as sole N source
Incubation time:	24 h	48 h	24 h	48 h	24 h	48 h	
Strain							
EMG50	−	−	−	−	−	−	++
Ahr 2–52	++	++	++	++	++	++	−
Ahr 2–8	++	++	++	++	++	++	−
Ahr 2–34	++	++	+	++	++	++	++
Ahr 2–48	++	++	+	++	++	++	++
Ahr 1–120	−	±	−	−	nt	nt	++
Ahr 2–9	++	++	+	++	++	++	++
Ahr 2–14	++	++	+	++	++	++	++
AM111	−	+	−	−	++	++	−

Strains were grown at 37° C on minimal salts – glucose agar containing the indicated compounds

−, no growth; ±, weak growth; +, fair growth; ++, good growth; nt = not tested

and AS activity is observed after growth in the presence of ornithine, which might exert this effect indirectly by depleting (through induction of arginase) the endogenous arginine pool that might be expected to repress these enzymes in unsupplemented medium.

Specific activities of the four arginine enzymes in the mutants representing classes 1, 2, 3 and 4 are also in general agreement with those observed by Harwood and Baumberg. Arginine biosynthesis in these four mutants seems largely unaffected. The mutants of classes 1 and 2, Ahr 2–52 and 2–8, have very low or undetectable arginase, and OAT is very weakly inducible by citrulline or ornithine. The class 3 mutant Ahr 2–34 showed no inducibility for arginase and OAT, as opposed to the diminished inducibility found by Harwood and Baumberg. The class 4 Ahr 2–48 mutant showed normal induction of arginase except in response to ornithine, and generally diminished induction of OAT. There were some discrepancies between the enzyme levels observed by Harwood and Baumberg for the remaining Ahr mutants and those recorded in Table 2. In the class 5 mutant Ahr 1–120, we found approximately wild-type specific activities for the biosynthetic enzymes whereas considerably elevated levels of these enzymes were reported previously. Both sets of data show slightly reduced inducibility of arginase and OAT. In the present experiments, class 6 mutants Ahr 2–9 and 2–14 exhibited non-inducibility of arginase while Harwood and Baumberg observed partial inducibility; OAT was partially inducible in both studies. These mutants are seen from Table 2 to have greatly elevated OCT levels with low repressibility, as noted for Ahr 2–14 by Harwood and Baumberg; Ahr 2–9 shows a similar effect for AS while this is wild-type in Ahr 2–14.

Although the reasons for these discrepancies are not clear, it may be relevant that over a year elapsed between the two sets of experiments. For instance, it seems likely from Tables 2 and 3 that Ahr 1–120 has reverted from the phenotype described by Harwood and Baumberg to one approaching wild-type. Accordingly, it was not felt worthwhile to continue investigation of this isolate.

A discrepancy was also observed for the specific activities of strain BC369. This strain carries a mutation *(argC4)* presumably in the structural gene for OCT, which showed no detectable activity in either study. The strain also carried *aut-1* conferring undetectable OAT activity, but whereas Harwood and Baumberg found wild type levels of arginase, Table 2 shows complete uninducibility for this enzyme. In the prototrophic derivative AM111, OCT activity is restored, OAT again being absent and arginase uninducible. *aut-1* is therefore similar to *ahr2–52* and *ahr2–8* in conferring very low levels of the catabolic

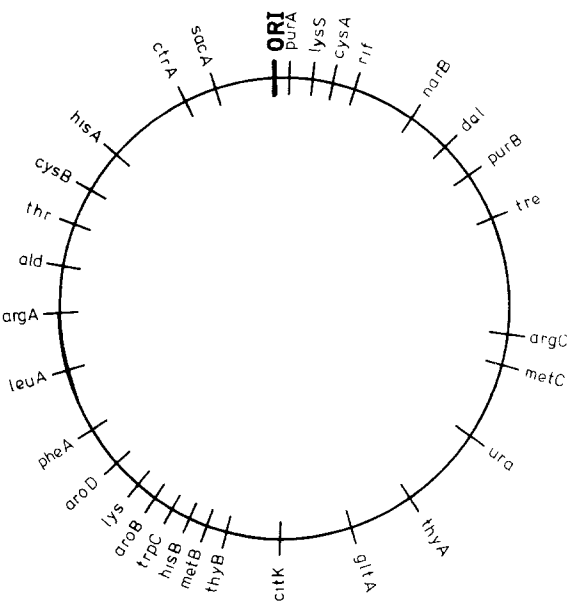

Fig. 1. The linkage map of *Bacillus subtilis*, showing loci utilised in this study (from Young and Wilson, 1976). ORI=Origin of replication

enzymes, and the plate tests recorded in Table 3 show that all three strains bearing these mutation are Out$^-$, while the other Ahr strains are Out$^+$. The strain AM111 harbouring *aut-1* is also Ahr, though only weakly – it grows on AH medium (100 µg/ml) after 48 h, while the mutants selected as Ahr (apart from Ahr 1–120 in this study) grow well on AH medium (200 µg/ml) after 24 h. These observations do not support the idea that *aut-1* is an OAT structural gene mutation but suggest instead that it is pleiotropic and therefore of the same nature as the other *ahr* mutations studied. Interestingly, the growth of AM111 on AH medium is strongly stimulated by ornithine, though citrulline does not have this effect. Under these conditions, growth of mutant Ahr 2–48 is strongly inhibited and the other Ahr's are unaffected. In this respect, AM111 resembles a new class of mutants to be designated Ahor, i.e. resistant to arginine hydroxamate in the presence of ornithine (Mountain and Baumberg, manuscript in preparation).

2. *Preliminary Mapping of the ahr Mutations*

An attempt was first made to locate roughly the various *ahr* mutations by contransduction with a set of 22 markers distributed more or less evenly around the *B. subtilis* genetic map (Fig. 1 and Table 1).

PBS1 lysates were prepared from the Ahr strains and AM111, and these were used to transduce the strains with markers listed in the second column of Table 1 to wild-type for each marker indicated. At least 100 transductants of each type were purified and screened for resistance to AH or (in the case of donor AM111) ability to use ornithine as sole nitrogen source. Markers *ahr2–52, 2–8,* and *2–48* showed cotransduction with *cysA14* (16% in all three cases) and *sacA321* (respectively 9%, 6% and 1%); *aut-1* with *cysA14* (14%); *ahr2–34* with *sacA321* (62%); and *ahr2–9* and *2–14* with *trpC2* (31% and 32%) and *hisB2* (22% and 23%). All other combinations of donor and recipient markers failed to give any cotransduction.

These results are largely consistent with those of Harwood and Baumberg (1977) in that as in their study *ahr2–52, 2–8* and *2–48* are found to be linked to *cysA14*, whereas *ahr2–14* is not. There is a discrepancy with *ahr2–34*, which Harwood and Baumberg found to be cotransducible with *cysA14*; we were unable to reproduce this.

3. Further Mapping of *ahr* Mutations Linked to *cysA14*

Additional two-point transductional crosses were performed to locate the mutations *ahr2–52, 2–8, 2–48* and *aut-1* with respect to markers either side of *cysA14*, namely *purA16, lysS1* and *rif-2*. Cotransduction with *purA16* and *lysS1* was measured as in Section 2 above, i.e. with the *ahr* mutations in the donor, and with *rif-2* by transducing the three Ahr strains and AM111 as recipients with the Rif^r strain MB26 as donor, selecting for Rif^r transductants. At least 100 transductants of each type were tested as above, except where *rif-2* was being selected: here Ahr 2–52 and 2–8 gave 60–80 transductants, while Ahr 2–48 and AM111 gave too few to use further.

All four markers showed higher cotransduction with *lysS1* (23–26%) than with *cysA14* (see above), and *ahr2–52* and *2–8* showed lower cotransduction with *rif-2* (10–11%). These results are consistent with the *ahr's* and *aut-1* lying to the *lysS1* and *purA16* side of *cysA14* rather than to its *rif-2* side. The results for cotransduction with *purA16* were however anomalous. *cysA14* and *purA16* are approximately 20% cotransducible (see e.g. Table 5 (a) below), so that on the above reasoning all four mutations should be closely linked to *purA16*. This prediction is fulfilled for *aut-1*, which shows 87% cotransduction with *purA16*. However, *ahr2–52* and *2–8* show only 16–19% cotransduction with *purA16*, and *2–48* only 6%. A possible explanation for this might have been reduced viability of Pur$^+$ transductants carrying the *ahr* markers; that an alternative explanation is necessary is indicated by results reported in (b) below.

The above results therefore suggest the arrangement *(purA16, ahr, aut-1) – lysS1 – cysA14 – rif-2.* Three-point crosses were then performed in order to refine this sequence.

(a) Three-Point Crosses Involving ahr, cysA14 and rif-2. An attempt was made to select spontaneous Rif^r derivatives of the Ahr strains; this was successful only for Ahr 2–52 and 2–8. The two resulting strains, Ahr 2–52 Rif^r and Ahr 2–8 Rif^r, were used as donors in transducing BD112 as recipient with selection for Cys$^+$ (Table 4(a)). Results are consistent either with the order *cysA14 – rif – ahr (2–52, 2–8)* or *ahr (2–52, 2–8) – cysA14 – rif*, the *ahr – cysA14* distance in the latter case being several times the *cysA14 – rif* distance.

(b) Three-Point Crosses Involving ahr, purA16 and cysA14. PBS1 lysates prepared from the Ahr strains and AM111 were used to transduce the *purA16 cysA14* recipient QB944 to either Cys$^+$ or Pur$^+$. Transductants were then tested for the unselected auxotrophic marker and for sensitivity/resistance to argi-

Table 4. Results of three-point transductional crosses (except those in which the selected marker was Pur$^+$)

(a) Crosses involving *ahr2–52* or *2–8, cysA14* and *rif* (donors, Ahr 2–52 Rif^r or Ahr 2–8 Rif^r; recipient, BD112; selected marker Cys$^+$)

	Donor *ahr* marker	
Unselected markers	*2–52*	*2–8*
Rif^{r}Ahr	31	28
Rif^{s}Ahr	0	0
Rif^{r}Ahs	184	188
Rif^{s}Ahs	7	6
Total	222	222

(b) Crosses involving *ahr2–52, 2–8, 2–48* or *aut-1, cysA14* and *purA16* (donors, Ahr 2–52, 2–8, 2–48 or AM111; recipient, QB944; selected marker Cys$^+$)

	Donor *ahr* marker			
Unselected markers	*2–52*	*2–8*	*2–48*	*aut-1*
Ahr^a Pur$^+$	21	6	3	17
Ahr Pur$^-$	0	0	0	0
Ahs Pur$^+$	10	4	4	9
Ahs Pur$^-$	117	88	94	122
Total	148	98	101	148

Table 4 (Continued)

(c) Crosses involving *ahr2–34*, *ctrA1* and *sacA321* (donor, Ahr 2–34; recipient, QB123; selected markers Ctr^+ or Sac^+)

Selected marker	Unselected markers	
Ctr^+	Sac^+ Ahr	110
	Sac^+ Ahs	0
	Sac^- Ahr	76
	Sac^- Ahs	36
Total		222
Sac^+	Ctr^+ Ahr	66
	Ctr^+ Ahs	0
	Ctr^- Ahr	20
	Ctr^- Ahs	62
Total		148

(d) Crosses involving *ahr2–9* or *2–14*, *aroD120* and *lys-1* (donors, Ahr 2–9 or Ahr 2–14, recipient, QB935; selected markers Aro^+ or Lys^+)

Selected marker	Unselected markers	Donor marker	
		ahr2–9	*2–14*
Aro^+	Lys^+ Ahr	4	2
	Lys^+ Ahs	0	0
	Lys^- Ahr	6	10
	Lys^- Ahs	212	210
Total		222	222
Lys^+	Aro^+ Ahr	8	4
	Aro^+ Ahs	0	0
	Aro^- Ahr	95	84
	Aro^- Ahs	119	97
Total		222	185

(e) Crosses involving *ahr* mutations in AM830Ahr-2, -7 or -33, *cysA14* and *purA16* (recipient, QB944; selected marker Cys^+)

Unselected markers	Donor strain		
	AM830 Ahr-2	-7	-33
Ahr[a] Pur^+	8	5	17
Ahr Pur^-	0	0	0
Ahs Pur^+	20	5	3
Ahs Pur^-	79	55	91
Total	107	65	111

[a] Ahr transductants were found also to show the Ahor/Ahos and Out^+/Aut^- phenotypes of the donor parents

nine hydroxamate (Ahs/Ahr); sensitivity/resistance to arginine hydroxamate in the presence of ornithine (Ahos/Ahor); and ability/inability to utilise ornithine as sole nitrogen source (Out^+/Out^-). The results are given in Tables 4(b) and 5(a).

Cys^+ transductants (Table 4(b)) with the donors Ahr 2–52 and 2–8 showed a perfect correlation between Ahr, Ahor and Out^- phenotypes; while, predictably, growth of Ahr transductants in the presence of AH was stimulated by ornithine when AM111 was the donor and inhibited when Ahr 2–48 was the donor (see Section 1 above). However, a more complex situation arose with Pur^+ transductants (Table 5(a)), in that (unlike Cys^+ transductants) some showed non-parental combinations of properties. For instance, with Ahr 2–52 and 2–8 as donors, some Pur^+ transductants were Ahr and Out^+, or Ahs and Out^-. Furthermore, whereas Cys^+ transductants showed an Ahr phenotype corresponding to the donor (i.e. for Ahr 2–52, 2–8 and 2–48 growth on AH medium within 24 h and for AM111 growth on AH medium containing ornithine within 24 h), some Pur^+ transductants with all four donors would only grow under these conditions within 48 h. For the Ahr 2–52 and 2–8 donors, ornithine accelerated somewhat the growth of those Ahr transductants appearing only after 48 h but did not change the overall pattern. For Ahr 2–48, only 41 Pur^+ transductants were obtained. None of these would grow on AH medium within 24 h, but the 32 that did so within 48 h were unaffected in this by the presence of ornithine. Another unexpected finding with Ahr 2–48 as donor was that some Pur^+ transductants were Out^-, whereas the Ahr 2–48 parent is Out^+ and shows only slightly reduced induction of arginine catabolic enzymes by ornithine (see Table 2). Both Ahr (48 h) Out^- and Ahs Out^- transductants appeared. With the donor AM111, non-parental phenotypes appeared among Pur^+ transductants only in regard to growth on AH medium in the presence of ornithine.

The origin of these non-parental phenotypes among Pur^+ but not Cys^+ transductants is not clear. Some possible interpretations will be considered in the Discussion. Nevertheless, if we consider the phenotype Ahr (48 h) Out^- alone as indicating the presence of the *ahr* or *aut-1* alleles and leave other combinations out of account, their linkages to *purA16* appear high: 86/111 (77%) for *ahr2–52*, 83/96 (86%) for *2–8*, 22/41 (54%) for *2–48*, and 92/111 (83%) for *aut-1*. These are more in line with those expected from the contransduction frequencies in two-point crosses with *cysA14*, *lysS1* and *rif-2* (see above), suggesting that the low cotransduction frequencies found in the two-point crosses with *purA16* reflect inadequate incubation time on AH medium.

The results with the Cys^+ transductants from the three-point crosses with QB944 as recipient (Table 4(b)) are consistent with the marker order *ahr*

Table 5. Results of three-point transductional crosses in which the selected marker was Pur$^+$

(a) Crosses involving *ahr2–52, 2–8, 2–48* or *aut-1*, *purA16* and *cysA14* (donors, Ahr 2–52, 2–8, 2–48 or AM111, recipient, QB944)

Unselected markers	Donor marker							
	ahr 2–52		*ahr 2–8*		*ahr 2–48*		*aut-1*	
Incubation time on AH medium:	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Ahr[a] Out$^-$[b] Cys$^+$	19	20	6	8	0	0	0	19[c]
Ahr Out$^+$ Cys$^+$	0	1	1	2	0	0	0	0
Ahr Aut$^-$ Cys$^-$	2	66	0	75	0	22	0	73
Ahr Out$^+$ Cys$^-$	0	1	0	3	0	10	0	0
Ahs Out$^-$ Cys$^+$		7		1		4		0
Ahs Out$^+$ Cys$^+$		7		2		3		2[d]
Ahs Out$^-$ Cys$^-$		2		0		0		0
Ahs Out$^+$ Cys$^-$		7		5		2		17
Total		111		96		41		111

[a] AH at 100 μg/ml
[b] Scored after 24 h
[c] Of these, 9 grew within 24 h on AH in the presence of ornithine (1000 μg/ml)
[d] Both of these grew within 48 h on AH in the presence of ornithine (1000 μg/ml)
Apart from the exceptions noted under [c] and [d], growth of all Pur$^+$ transductants on AH was not affected by the presence of ornithine

(b) Crosses involving *ahr* mutations in AM830Ahr-2, -7 or -33, *purA16* and *cysA14* (recipient, QB944); selected marker Pur$^+$)

Unselected markers	Donor strain					
	AM830 Ahr-2		*−7*		*−33*	
Incubation time on AH medium:	24 h	48 h	24 h	48 h	24 h	48 h
Ahr Out$^-$[a] Cys$^+$	0	0	0	0	15	18
Ahr Out$^+$ Cys$^+$	7	7	4	7	2	2
Ahr Out$^-$ Cys$^-$	0	48	0	51	2	63
Ahr Out$^+$ Cys$^-$	0	2	0	0	0	2
Ahs Out$^-$ Cys$^+$		7		1		21
Ahs Out$^+$ Cys$^+$		6		5		2
Ahs Out$^-$ Cys$^-$		2		2		0
Ahs Out$^+$ Cys$^-$		1		1		3
Total		73		67		111

[a] Scored after 24 h

or *aut-1 – purA16 – cysA14* but not with the alternative *purA16 – cysA14 – ahr* permitted by the results in Table 4(a). Hence the results suggest the marker order *sacA–ahr–purA–lysS–cysA–rif* for the markers *ahr2–52, 2–8, 2–48* and *aut-1*. No unambiguous order of these *ahr* markers emerges and they have not yet been mapped directly against one another. It is proposed to designate the locus defined by these mutations as *ahrA*.

4. Further Mapping of the sacA321-Linked ahr2–34

The transductional cross involving Ahr 2–34 as donor and QB123 as recipient was repeated. On this occasion both Ctr$^+$ and Sac$^+$ transductants were selected, and the two unselected markers scored (Table 4(c)). The marker order *ctrA1–ahr2–34–sacA321* is unambiguously indicated, *ahr2–34* showing 84% cotransduction with *ctrA1* and 58% with *sacA321*. It is proposed to designate the locus defined by *ahr2–34* as *ahrB*.

5. Further Mapping of the trpC2- and his B2-Linked ahr2–9 and 2–14

Two- and three-point transductional crosses were performed with Ahr 2–9 and 2–14 as donors and as recipients SB202 carrying the *aroB*, AM210 carrying the *metB5*, and QB935 carrying the *aroD120* and *lys-1* markers.

Crosses with Ahr 2–8 or 2–14 as donor and SB202

Table 6. Properties of Ahr derivatives of AM830 without and with the suppressor *sup-1*

Strain	Growth (37° C, scored after 42 h) on minimal salts-glucose medium			Sensitivity to phages		Specific activities of arginine enzymes					
						Minimal salts-glucose medium − arginine			Minimal salts-glucose medium + arginine		
	cysteine	cysteine + threonine + histidine	cysteine + threonine + histidine + AH	SPO1	SPO1 -F51	A[a]	OAT	OCT	A	OAT	OCT
AM830	−	++	−	S[b]	R	7.0	0.44	4.6	43	>6	u[c]
AM830 Ahr2	−	++	++	S	R	3.5	0.30	13.1	7.2	0.77	u
AM830 Ahr7	−	++	+	S	R	7.0	0.55	8.2	75	>6	0.6
AM830 Ahr30	−	++	++	S	R	7.0	0.55	2.0	81.5	1.5	u
AM830 Ahr33	−	++	++	S	R	u	u	1.9	u	u	0.8
AM830 Ahr37	−	++	+	S	R	4.2	0.49	6 6	67	>6	u
AM830 Ahr2 *sup-1*	++	++	++	S	S	4.6	0.28	8.7	14	1.2	u
AM830 Ahr7 *sup-1*	++	++	−	S	S	4.3	0.40	5.6	88	>6	u
AM830 Ahr30 *sup-1*	++	++	−	S	S	3.6	0.31	6.5	80	2.3	u
AM830 Ahr33 *sup-1*	++	++	++	S	S	u	u	8 9	u	u	0.9
AM830 Ahr37 *sup-1*	++	++	−	S	S	4.8	0.47	8.5	17	>6	u

AM830, five Ahr derivatives, and presumed *sup-1*-containing Thr$^+$ His$^+$ transductants thereof (the donor being HA101B, *leuA8 metB5 (sus) hisB5 (sus) sup-1*) were tested for: growth on minimal salts-glucose media supplemented with cysteine alone, cysteine + threonine + histidine, or cysteine + threonine + histidine + AH (250 µg/ml); sensitivity or resistance to phage SPO1 and its *sup-1* suppressible derivative F51; and specific activities of the enzymes arginase, OAT and OCT. For details of the latter, see legend to Table 2 and Materials and Methods

[a] A = arginase
[b] S = sensitive; R = resistant
[c] u = unmeasurably small

as recipient showed 32% (Ahr 2–9) and 28% (Ahr 2–14) linkage of the *ahr* mutation to *aroB*, and with AM210 as recipient showed 3% (Ahr 2–9) and 4% (Ahr 2–14) linkage to *metB5* (70–75 Aro$^+$ or Met$^+$ transductants tested from each cross). The results of three-point crosses with QB935 are given in Table 4(d), and suggest the order *aroD120 – (ahr2–9, 2–14) – lys-1*. These findings together with the preliminary mapping in Section 2 above indicate an arrangement *aroD120 – (ahr2–9, 2–14) – lys-1/–21 – (aroB, trpC2) – hisB2 – metB5*. Percent cotransductions, taking *ahr2–9* and *2–14* together, are: *aroD120* 5%; *lys-1/–21* 47%; *aroB* 30%; *trpC2* 32%; *hisB2* 22%; and *metB5* 3%. It is proposed to designate the locus defined by *ahr2–9* and *2–14* as *ahrC*.

6. *Nature of a Functional Product Specified by the ahrA Locus*

A preliminary attempt was made to ascertain whether the functional product of some or all of the *ahr* loci are proteins. The method employed was first to isolate Ahr derivatives of strain AM830, which carries two non-cotransducible markers, *thr-5* and *hisB5*, known both to be suppressed by the suppressor *sup-1* (Tevethia et al., 1974). 40 Ahr mutants of AM830 were independently isolated (i.e. from separately inoculated cultures) and transduced with a PBS1 lysate prepared on strain HA101B (grown on minimal salts-glucose + leucine + 0.004% tryptone) which carries *sup-1* (Okubo and Yanagida, 1968; Tevethia et al., 1974), transductants being selected simultaneously for Thr$^+$ and His$^+$. That these had indeed received *sup-1* was demonstrated by their sensitivity to mutant F51 of phage SP01, which carries a mutation known also to be suppressed by *sup-1* (Tevethia et al., 1974). The sensitivity/resistance to arginine hydroxamate of the *sup-1* transductants was then compared with that of the original mutants. Table 6 shows that three of the 40 Ahr derivatives became Ahs as a result of acquiring *sup-1*, namely AM830Ahr-7, -30 and -37 (data for two others, AM830Ahr-2 and -33, are included for comparison). Levels of three arginine enzymes (arginase, OAT and OCT) were measured in frozen and thawed lysates of these five Ahr mutants and of one *sup-1*-carrying transductant derived from each (Table 6). It appears that the three suppressible Ahr mutants show wild-type inducibility of arginase and OAT and, predictably, introduction of *sup-1* into these strains has no effect on these enzyme activities. These Ahr mutants may correspond to the class 4 mutants of Harwood and Baumberg (1977), and in this respect it is interesting that all three show the inhibition by

ornithine of growth on AH medium exhibited by Ahr 2–48, which also falls in this class (Mountain and Baumberg, manuscript in preparation).

Two- and three-point crosses were performed in order to assign the AM830 *ahr* mutations to one of the loci *ahrA, B* or *C*. The five Ahr mutants listed in Table 6 were used as donors in PBSl-mediated transductional crosses with strains QB944 *(purA16 cysA14)*, QB123 *(sacA321)* and QB935 *(aroD120 lys-1)* as recipients, selecting for transduction to wild-type of the markers shown in brackets. Transductants were screened for resistance to AH and for the unselected nutritional marker when QB944 was used as recipient. AM830Ahr-2, -7 and -33 all carry mutations in *ahrA*, as shown by cotransduction of Ahr with Cys^+ (7%, 8% and 15% respectively) and Pur^+ (10%, 6% and 17% respectively), 65–115 transductants being tested in each case. AM830Ahr-33 also gave 5% (5/100) cotransduction of Ahr with Sac^+; otherwise, no cotransduction was observed. Analysis of unselected markers in Cys^+ transductants (Table 4(e)) gave the marker order *ahr – purA16 – cysA14* as indicated by the absence of an Ahr Pur^- class. The Pur^+ transductants show the same anomalous appearance of non-parental phenotypes (Ahr Out^+, Ahs Out^-, and Ahr manifest only after 48 h whereas the AM830Ahr's were all originally selected on AH medium after 24 h; Table 5(b)) as described in Section 3 above for crosses involving Ahr 2–52, 2–8, 2–48 and AM111. Because AM830Ahr-30 and -37 consistently gave poor transducing lysates, their *ahr* mutations have not yet been properly mapped; however, the appearance of one Ahr Pur^+ transductant with AM830Ahr-37 as donor suggests that this isolate also carries an *ahrA* mutation. The suppressibility by *sup-1* of the Ahr phenotype in AM830Ahr-7, which evidently carries a mutation in *ahrA*, suggests that this locus encodes at least one functional polypeptide product.

Discussion

The mutations conferring arginine hydroxamate resistance in seven mutants isolated by Harwood and Baumberg (1977) have been mapped by PBS1-mediated transduction. The seven mutants represent five of the six phenotypic classes described by Harwood and Baumberg. Mutants Ahr 2–52, 2–8, 2–34 and 2–48, representing classes 1, 2, 3 and 4 respectively, are affected only in expression of the arginine catabolic enzymes arginase and OAT; while mutants Ahr 2–9 and 2–14, both representatives of class 6, in addition to similar effects on these catabolic enzymes also show elevated expression and greatly reduced repressibility of at least some arginine biosynthetic enzymes. A seventh mutation, *aut-1*, originally present in a strain BC369 not selected as Ahr, was also investigated. It was found to confer weak arginine hydroxamate resistance and to affect expression only of arginine catabolic enzymes.

The mapping experiments suggest that mutations within at least three loci can confer arginine hydroxamate resistance. Mutations *ahr2–52, 2–8* and *2–48*, together with *aut-1*, are located in the vicinity of the replication origin at a locus designated *ahrA*. The results of two- and three-point crosses indicate the order *sacA–ahrA–purA–lysS–cysA–rif*. A curious anomaly was observed in that some transductants selected as Pur^+, but not those selected as Cys^+, with these Ahr donors showed phenotypes intermediate between the parents; whereas the donors were Ahr and Out^-, and the recipient Ahs and Out^+, a minority of the Pur^+ transductants were Ahr and Out^+, or Ahs and Out^-. Furthermore, while the donors (except AM111) could grow on AM medium in 24 h, some Pur^+ transductants took 48 h to grow in these circumstances. No consistent explanation has been found for these phenomena, although formally one may postulate the existence of at least two modifier loci sufficiently far to the left of all the *ahrA* mutations that they are never cotransduced with $cysA^+$. It might be that AH under the conditions originally employed (Harwood and Baumberg, 1977) selects for successive mutations, so that the Ahr mutants differ at more than one site from the parental Ahs. One such mutation in each of the four Ahr strains may be cotransducible with *cysA* and also have the major effect in that it confers the phenotype of the Ahr parent in Cys^+ transductants; the modifier mutations, affecting this phenotype, might be located to the left of *ahr2–48* – already only poorly cotransducible with $cysA^+$. The problem with this kind of explanation is why the 'major' mutations should reproduce the parental phenotype when separated from the modifiers in Cys^+ transductants? It is hard to avoid calling upon strain background effects to overcome this difficulty.

An attempt was made to determine the origin of the non-parental phenotypes among transductants selected as Pur^+ by measuring specific activities of arginase and OAT in arginine-supplemented cultures of representatives of the six possible classes of Pur^+ transductants (the Cys character being ignored) from crosses with donors carrying *ahr2–52, 2–8* and *aut-1*. The results (Mountain and Baumberg, unpublished data) unfortunately did not clarify the situation, in that there was only imperfect correlation between the Ahr/s and Out^-/Out^+ phenotypes and enzyme levels. However, it was the case that only Ahr transductants (whether within 24 or 48 h) showed the uninducibility

of the donor parent; those that were Ahr (within 24 h) Out$^-$ were always uninducible; and those that were Ahs Out$^+$ were always inducible as in the wild-type.

The single mutation *ahr2–34* in class 3 was found to be cotransducible with *sacA321* and *ctrA1*, but not with *cysA14* as reported by Harwood and Baumberg (1977). This mutation is taken to define an *ahrB* locus. Three-point crosses clearly indicated the order *ctrA–ahrB–sacA*.

The mutations *ahr2–9* and *2–14*, which confer the class 6 phenotype, simultaneously reduce expression of arginine catabolic enzymes and increase expression of biosynthetic enzymes. Both were found to be co-transducible with *aroD120* and *lys-1/–21*, and hence define a third locus *ahrC*. Three-point crosses provide the order *aroD–ahrC–lys*.

A preliminary attempt was made to define the nature of the active products encoded by the *ahr* loci. 40 Ahr mutants were independently isolated from a strain carrying the cosuppressible *thr-5* and *hisB5* mutations and *sup-1*, which is known to suppress both these markers, was transduced in. Of the 40 strains tested, three became Ahs as a result of acquiring *sup-1*, and since there is evidence that this is a conventional nonsense suppressor (Baptist et al., 1974; Shub, 1975) it would appear that the gene(s) in which these three *ahr* mutations occur encodes a functional polypeptide. Preliminary mapping experiments suggest that at least one of these mutations lies in *ahrA*.

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