Relationship Between the Axenic Phenotype and Sensitivity to ω -Aminocarboxylic Acids in *Dictyostelium discoideum*

By MICHAEL J. NORTH

Department of Biochemistry, University of Stirling, Stirling FK9 4LA, Scotland

AND KEITH L. WILLIAMS

*Department of Genetics, Research School of Biological Sciences,
The Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601,
Australia*

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A previous suggestion that sensitivity to ω -aminocarboxylic acids in *Dictyostelium discoideum* is associated with the ability to grow axenically has been re-evaluated using new axenic strains and axenic derivatives of strain AX3 recombinant for linkage group II. It is now shown that the major gene for ω -aminocarboxylic acid sensitivity, designated *oaaA*, is not allelic to previously described axenic loci *axeA* and *axeB*, but that *oaaA* is closely linked to, or allelic with a third axenic gene *axeC* found in strains AX2 and AX3. The *axeC* phenotype is rapid growth in axenic medium. The *oaaA* locus is located on linkage group II. Genetic evidence suggests that the strains AX2 and AX3 now in existence share a common genetic background and may not represent independent isolates from the wild-type strain NC4. Studies with ^{14}C -labelled ϵ -aminocaproic acid show that sensitivity to ω -aminocarboxylic acids is correlated with increased myxamoebal permeability to these compounds.

INTRODUCTION

Biochemical studies of the cellular slime mould *Dictyostelium discoideum* have been greatly enhanced in recent years by the availability of strains capable of growth in axenic medium (Sussman & Sussman, 1967). Two strains in particular have been used, AX2 (Watts & Ashworth, 1970) and AX3 (Loomis, 1971), both derived ultimately from the wild-type strain NC4. Despite the widespread use of these strains, the biochemical properties which permit them to grow on nutrients other than bacteria are not known. In many respects, and not least in their ability to undergo fruiting body formation, they do not differ from wild-type strains. However it was recently observed that AX2 and AX3 both differ from the wild type with respect to their sensitivity to ω -aminocarboxylic acids, agents which block post-aggregation development of sensitive strains (North & Ashworth, 1976). A use for this property in investigating the molecular basis of the ability to grow axenically was suggested.

Axenicity is a recessive character (Williams *et al.*, 1974*a*). Complementation studies have indicated that the axenic phenotype has the same genetic basis in AX2 as it does in AX3 (Williams *et al.*, 1974*a*), and on the basis of linkage analysis it has been suggested (Williams *et al.*, 1974*b*) that only two mutations are involved, in gene *axeA* on linkage group II and gene *axeB* on linkage group III. Like the ability to grow axenically, sensitivity to ω -amino acids is recessive (North & Ashworth, 1976), and a study involving a number of strains showed a close correlation between the two characters. It was suggested that one of the axenic genes, *axeA*, might confer sensitivity to ω -amino acids, and that a second gene, possibly *axeB*, might be required for the maximum sensitivity displayed by AX2 and AX3 (North & Ashworth, 1976).

Table 1. *Description of Dictyostelium discoideum strains used*

Strain	Parent(s)	Genotype*	References
NC4	—	Wild type	Raper (1935)
v12	—	Wild type	Erdos <i>et al.</i> (1973)
AX2	NC4	<i>axeA2, axeB2, axeC2, oaaA2</i>	Watts & Ashworth (1970)
AX3	NC4	<i>axeA1, axeB1, axeC1, oaaA1</i>	Loomis (1971)
NP73	v12	<i>axe-352</i>	Williams (1976)
NP82	NP20	<i>axe-351, cyc-5</i>	Williams (1976)
NP84	NP73	<i>axe-352, tsG-350</i>	Mosses <i>et al.</i> (1975)
x11†	DP19	<i>axeA1, axeB1, acrA1, tsG1, whiA1, bwnA1</i>	Williams <i>et al.</i> (1974b)
HN1	DP49	<i>axeA1, axeC1, acrA9, oaaA1</i>	M. J. North, methanol-resistant haploid from strain DP49
DP22	NP16/x9	<i>axeA1, axeB1, axeC1, oaaA1, tsG3/acrA1, cycA1, tsGD12, whiA1, bwnA1</i>	K. L. Williams (unpublished)
DP27	NP16/x2	<i>axeA1, axeB1, axeC1, oaaA1, tsG3/axeA1, axeB1, axeC1, oaaA1, tsG1, bwnA1</i>	Brody & Williams (1974)
DP29	NP16/NP24	<i>axeA1, axeB1, axeC1, oaaA1, tsG3/axeA2, axeB2, axeC2, oaaA2, tsG-14</i>	Brody & Williams (1974)
DP30	NP24/x9	<i>axeA2, axeB2, axeC2, oaaA2, tsG-14/acrA1, cycA1, tsGD12, whiA1, bwnA1</i>	Williams <i>et al.</i> (1974b)
DP49	TS12/NP59	<i>cycA1, tsGD12, whiA1/axeA1, axeB1, axeC1, oaaA1, acrA9, tsG1, bwnA1</i>	Williams <i>et al.</i> (1974b)

* Standard nomenclature based on that used in bacterial genetics (Demerec *et al.*, 1966): *axe*, growth in axenic medium if *axeA* and *axeB* are present (genetic basis of axenic growth in NP73, NP82 and NP84 not yet determined); *acr*, acriflavin resistance; *cyc*, cycloheximide resistance; *tsG*, temperature-sensitive growth; *whi*, white fruiting bodies; *bwn*, brown pigment production; *oaa*, development sensitive to ω -aminocarboxylic acids. Only mutant alleles are shown.

† Strain x11 almost certainly contains *tsGD12* in addition to *tsG1*.

The recent isolation of new axenic strains derived completely independently of AX2 and AX3 (Williams, 1976) has allowed us to extend these observations on the relationship between the axenic phenotype and ω -amino acid sensitivity. We report here that some strains capable of growth in axenic medium are not sensitive to the ω -amino acids, ϵ -aminocaproic acid (EACA) and *p*-aminomethylbenzoic acid (PAMBA). However, there is a correlation between a strain's rate of growth in axenic medium and its sensitivity. In an attempt to understand the biochemical basis of ω -amino acid sensitivity we have examined the uptake of radioactively labelled EACA and demonstrated a correlation between sensitivity and permeability to EACA.

METHODS

Chemicals. *p*-Aminomethylbenzoic acid, supplied as a solid, was a gift from VEB Arzneimittelwerk, Dresden, DDR. ϵ -Aminocaproic acid, bovine albumin (fraction V) and cycloheximide were supplied by Sigma; sodium azide was from BDH; and ϵ -amino[1-¹⁴C]caproic acid was supplied by New England Nuclear. All other chemicals were of the highest purity commercially available.

Strains. The strains of *Dictyostelium discoideum* used in this study are described in Table 1. The maintenance of stocks on bacteria (*Klebsiella pneumoniae*) and growth conditions have been described previously (Williams *et al.*, 1974b).

Growth in axenic medium. Myxamoebae were grown in 70 ml batches of HL5 medium containing 86 mM-glucose at 22 °C and 130 rev. min⁻¹ in 250 ml Erlenmeyer flasks (Watts & Ashworth, 1970). Cell densities were determined with a haemocytometer.

Sensitivity of strains to ω -aminocarboxylic acids. This was determined as described previously (North & Ashworth, 1976) by testing the cells' ability to form fruiting bodies either after growth with *K. pneumoniae* on SM agar plates (Sussman, 1966) or after deposition and incubation of washed cells on Selectron filters (AG91/3; Anderman & Co., East Molesey, Surrey) over Whatman glass-fibre pads (GF/D) saturated with pad diluting fluid (PDF; 25 mM-KCl, 5 mM-MgCl₂, 50 mM-sodium potassium phosphate buffer, pH 6.5). For both tests EACA or PAMBA were present at final concentrations of 50 mM and 10 mM, respectively.

Table 2. *Axenic phenotype, genotype and sensitivity to EACA and PAMBA*

Strain	Axenic phenotype	Genotype	Sensitivity*
NC4	Non-axenic	+	R
v12	Non-axenic	+	R
AX2	Axenic	<i>axeA2, axeB2, axeC2</i>	S
AX3	Axenic	<i>axeA1, axeB1, axeC1</i>	S
NP73	Axenic	<i>axe-352</i>	R
NP82	Axenic	<i>axe-351</i>	R
NP84	Axenic	<i>axe-352</i>	R
x11	Axenic	<i>axeA1, axeB1,</i> <i>axeA1, axeC1</i>	R
HN1	Non-axenic	<i>axeA1, axeC1</i>	S
DP22	Non-axenic	<i>axeA1, axeB1, axeC1/+ + +</i>	R'
DP27	Axenic	<i>axeA1, axeB1, axeC1/axeA1, axeB1, axeC1</i>	S
DP29	Axenic	<i>axeA1, axeB1, axeC1/axeA2, axeB2, axeC2</i>	S
DP30	Non-axenic	<i>axeA2, axeB2, axeC2/+ + +</i>	R'

* R, Resistant, formed fruiting bodies in the presence of 50 mM-EACA or 10 mM-PAMBA at 24 °C. R', Resistant, but formed slightly abnormal fruiting bodies. S, Sensitive, failed to form fruiting bodies in the presence of 50 mM-EACA or 10 mM-PAMBA at 24 °C.

Uptake of [1-¹⁴C]EACA. This was examined using two methods. In the first, 0.5 ml suspensions of myxamoebae were deposited on Selectron filters at a density of 5×10^7 to 10×10^7 myxamoebae per filter over pads saturated with 1.6 ml PDF containing 65.5 mM-[1-¹⁴C]EACA (0.38 μ Ci mmol⁻¹). After 12 or 24 h incubation at 24 °C, myxamoebae were harvested into 10 ml PDF containing 50 mM unlabelled EACA and collected by centrifugation (1 min, 500 g). The myxamoebae were resuspended in a further 10 ml PDF plus 50 mM-EACA, collected by centrifugation and finally resuspended in 0.5 ml water. Then 0.5 ml 10 % (w/v) trichloroacetic acid was added and the sample was centrifuged (5 min, 500 g). The supernatant was retained and the pellet washed with 1 ml 5 % (w/v) trichloroacetic acid. After centrifugation, the supernatants were combined and transferred to a scintillation vial with 10 ml of scintillation mixture [31.5 g 2(4'-*tert*-butyl-phenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole, 500 ml methanol, 3 l toluene, 1.5 l Triton X-100] and counted for radioactivity in a Philips liquid scintillation analyser. Measurements were corrected to d.p.m. by the channels-ratio method. The acid-insoluble material was dissolved in 2 M-NaOH and protein was determined by the method of Lowry *et al.* (1951) using bovine albumin as standard.

In the second method, washed myxamoebae were resuspended in 10 ml 17 mM-sodium phosphate buffer, pH 6.0, at a density of 5×10^6 to 10×10^6 myxamoebae ml⁻¹. The myxamoebae were shaken at 24 °C and 130 rev. min⁻¹ for 1 h (unless otherwise indicated) and then [1-¹⁴C]EACA was added to a final concentration of 50 mM (2.22 μ Ci mmol⁻¹). At intervals, 1 ml samples of cell suspension were removed and the cells were collected by centrifugation (1 min, 500 g), washed twice with 17 mM-sodium phosphate buffer, pH 6.0, plus 50 mM unlabelled EACA and then resuspended in 1 ml water. Radioactivity in the sample was measured as above. Other samples were removed, and trichloroacetic acid was added to a final concentration of 5 % (w/v) and the protein content of the insoluble material was determined as above.

RESULTS

Sensitivity of strains to EACA

The development of the wild-type (non-axenic) strains NC4 and v12 was insensitive to EACA and PAMBA in contrast to that of the axenic strains AX2 and AX3 (Table 2). Three new axenic strains NP82, NP73 and NP84, derived from NC4 or v12, were also insensitive to EACA and PAMBA (Table 2). Clearly sensitivity to EACA and the ability to grow axenically are not correlated as had been suggested previously (North & Ashworth, 1976). Sensitivity to EACA, the primary genetic determinant of which shall be designated *oaaA*, was separated from the axenic genotype (*axeA* and *axeB*) of strain AX3 in the axenic strain x11 which is resistant to EACA (Table 2). Strain x11 is an *oaaA*⁺ recombinant for linkage group II, carrying the mutations *whiA1* and *tsgD12* from a non-axenic parent (strain NP14) and *axeA1* from the axenic parent (NP46, derived from AX3) (Williams *et al.*, 1974b).

Sensitivity to EACA was shown to be incompletely recessive to wild type and indistinguishable in strains DP22 (AX3 $\times$ non-axenic) and DP30 (AX2 $\times$ non-axenic), which are

Table 3. *Growth of axenic strains in HL5 medium*

Cells were grown in two 70 ml batches of medium (experiments 1 and 2). The doubling time is given in terms of cell numbers.

Strain	Doubling time (h)	
	Expt 1	Expt 2
AX2	13	—
AX3	14	9
NP82	42	25
NP84	44	38
x11	47	42
NP73	54	38

heterozygous for EACA sensitivity and form fruiting bodies in the presence of 50 mM-EACA at 24 °C which are slightly abnormal (Table 2). Two similar diploids, DP49 and DP59, which were previously reported (North & Ashworth, 1976) to be fully resistant to EACA, behaved similarly. A diploid, DP27 (Table 2), which was homozygous for axenic genes from AX3, was as sensitive to EACA as were the haploid parents. Examination of strain DP29 (AX3 × AX2 diploid) showed that the EACA sensitivity of strains AX2 and AX3 is allelic since there was no complementation.

Definition of axenicity

In previous genetic studies on axenic strains of *D. discoideum*, axenicity was defined as the ability to reach a cell density of greater than 2×10^6 amoebae ml⁻¹ in axenic medium at stationary phase (Williams *et al.*, 1974*b*). This definition of axenicity does not take into account the rate of growth in the medium. In those previous studies, axenic genes were located on linkage groups II and III in strain AX3 and mutations at both sites were required for axenic growth (Williams *et al.*, 1974*b*). More recently, when axenic strains recombinant for linkage group II were analysed (e.g. x11), a new axenic phenotype was observed. This was a slower growth rate than is usual in strains AX2 and AX3. The phenomenon is most easily explained by proposing the existence of at least one locus (*axeC*) in addition to *axeA* on linkage group II. The phenotype of *axeC* is rapid growth in axenic medium when *axeA* and *axeB* are also present. Table 3 shows the growth rates of the axenic strains studied here in two batches of axenic medium. While doubling times as low as 20 h could be achieved by the new axenic strain NP82, only strains AX2 and AX3 exhibited a doubling time of less than 15 h in axenic medium. Thus the phenotype of the mutation at *axeC* can be defined as producing a doubling time of less than 15 h. The *axeC* phenotype is suppressed in some AX3-derived strains, e.g. strain NP7 (Kessin *et al.*, 1974). Strain x20 (AX3-derived; Williams *et al.*, 1974*b*) also grows poorly in axenic medium, but this may be due to a general defect in growth rate since growth in association with *K. pneumoniae* is also slow in this strain.

Relationship between growth rate in axenic medium and EACA sensitivity

Examination of Tables 2 and 3 shows that only strains AX2 and AX3, which carry the proposed new locus *axeC*, are EACA sensitive (i.e. carry *oaaA*). Thus EACA sensitivity and fast axenic growth are correlated, although more detailed studies are needed to see whether the *axeC* and *oaaA* loci can be separated genetically. It is relevant to point out that two axenic strains, x20 and NP7, in which *axeC* is suppressed, remained EACA sensitive; the *oaaA* phenotype is still expressed.

Permeability of myxamoebae to EACA

Permeability of strain AX2. When axenically grown myxamoebae were incubated in phosphate buffer containing [1-¹⁴C]EACA, radioactivity was recovered associated with cells washed well with unlabelled EACA. Over 99% of the radioactive material was soluble

Fig. 1

Fig. 2

Fig. 1. Uptake of EACA by Ax2 myxamoebae in the presence of (a) 500 μg cycloheximide ml^{-1} and (b) 1.0 mM-sodium azide. Washed myxamoebae were incubated for 5 h in 17 mM-sodium phosphate buffer, pH 6, and then 50 mM-[1- ^{14}C]EACA (2.22 $\mu\text{Ci mmol}^{-1}$) was added (zero time). Inhibitor was added 1 h after EACA (●), 30 min before EACA (□) or 5 h before EACA (■); no inhibitor added (○).

Fig. 2. Uptake of EACA by Ax2 myxamoebae on filters. Washed myxamoebae were deposited at 0 h and sets of filters were transferred to pads containing 50 mM-[1- ^{14}C]EACA (0.38 $\mu\text{Ci mmol}^{-1}$) at 0 h (○), 4 h (●), 8 h (□), 12 h (■) and 16 h (△).

in 10% (w/v) trichloroacetic acid and co-migrated with authentic EACA on paper chromatograms. As in other systems (Hargreaves & Evered, 1973; Chou *et al.*, 1974), there was no evidence that EACA was metabolized.

The initial rate of EACA uptake was directly proportional to the concentration of EACA in the buffer over the range 0.08 to 50 mM. Uptake of radioactivity was linear for approximately 1 h and then slowed to give a maximum level after 2 to 4 h (Fig. 1). This level was proportional to the extracellular EACA concentration, and the maximum amount of radioactivity associated with the cells indicated an intracellular concentration of the same order as that of the buffer.

The labelling of cells was inhibited by cycloheximide, which prevented uptake if added before the EACA, and by sodium azide, which had an immediate effect (Fig. 1). A similar effect to that of sodium azide was observed with 0.1 mM-dinitrophenol. The effects of cycloheximide and dinitrophenol differed from those reported by Lee (1972) on lysine uptake by Ax2 myxamoebae. However, our own observations indicate that the uptake of lysine is inhibited by cycloheximide, dinitrophenol and sodium azide (M. J. North, unpublished). In *Acanthamoeba castellanii*, leucine uptake is believed to occur by pinocytosis (Bowers & Olszewski, 1972), a process that is sensitive to inhibitors of oxidative metabolism (Bowers, 1977). The inhibitory effects observed with *D. discoideum* suggest that EACA uptake may also be dependent on pinocytosis.

Uptake of EACA was also demonstrated in myxamoebae incubated on filters. The rate and extent of uptake (on a total cell protein basis) did not vary significantly when EACA was given at different stages of development (Fig. 2).

Permeability of other strains. Washed myxamoebae, grown with *K. pneumoniae* on SM agar, were deposited on filters and allowed to develop in the presence of [1- ^{14}C]EACA. Under these conditions, the uptake of EACA correlated with sensitivity to the agent (Table 4). During the incubation period, sensitive axenic strains, both haploid and diploid, took up most EACA. Much less radioactivity was associated with cells of the resistant axenic strains; uptake was the same as with non-axenic strains. Three diploids, DP22, DP30

Table 4. *Uptake of [1-¹⁴C]EACA during incubation on Selectron filters*

Bacterially grown myxamoebae (0.5 ml) were deposited on Selectron filters over pads saturated with pad diluting fluid (1.6 ml) containing 65.5 mM-[1-¹⁴C]EACA (0.38 μ Ci mmol⁻¹). The EACA associated with the cells was estimated after incubation at 24 °C for 12 or 24 h.

Strain	EACA associated with cells [nmol (mg protein) ⁻¹]	
	12 h on filters	24 h on filters
AX3	930	780
DP29	573	580
DP27	572	922
AX2	515	760
HN1	430	332
DP30	360	622
DP49	242	230
DP22	232	588
NP73	147	89
V12	141	119
X11	119	105
NC4	118	98
NP82	112	99
NP84	107	58

Fig. 3. Uptake of EACA by axenically grown myxamoebae in 17 mM-sodium phosphate buffer, pH 6. Washed myxamoebae were shaken for 1 h before addition of 50 mM-[1-¹⁴C]EACA (2.22 μ Ci mmol⁻¹) at zero time.

and DP49, which were heterozygous for both the axenic genes and those for ω -amino acid sensitivity, took up more EACA than the resistant haploids. The presence of higher intracellular concentrations of EACA than in resistant haploid strains probably accounts for the formation of slightly abnormal fruiting bodies by these strains. An intermediate level of uptake of EACA was also observed with strain HN1 (linkage group II from AX3 and linkage group III from NC4). All strains with this genotype are sensitive, although in the presence of 50 mM-EACA at 24 °C development can proceed beyond aggregation with the formation of finger-like structures as the final form (North & Ashworth, 1976).

Similar results were obtained when washed bacterially grown myxamoebae were shaken in sodium phosphate buffer containing [1-¹⁴C]EACA. However, there was more variation between different sets of data from the same strain, and certain strains displayed uptake behaviour that differed slightly from that observed on filters. The ω -amino acid resistant

strains NC4, x11, NP73, NP82 and NP84 all took up very little EACA in comparison with the other strains. Of the ω -amino acid sensitive axenic strains, the diploids DP27 and DP29 consistently took up more EACA than the haploid strains AX2 and AX3, both on a cell number basis and on a total cell protein basis. The reason for this was not clear. The heterozygous diploids DP22 and DP30 often took up more EACA under these conditions than they did on filters, particularly DP30.

The variation observed in the above experiments may have been the result of variable residual numbers of bacteria that were not removed despite thorough washing and pre-incubation. More consistent results were obtained with axenically grown cells. The results in Fig. 3 demonstrate the difference between the sensitive axenic strains and the resistant axenic strains in their ability to take up EACA.

DISCUSSION

The suggestion by North & Ashworth (1976) of a link between the ability of a strain to grow axenically and its sensitivity to ω -aminocarboxylic acids was based on the finding that two strains, AX2 and AX3, reported to be isolated independently for their ability to grow in axenic medium, were both unable to develop in the presence of ω -amino acids, in contrast to the behaviour of wild-type non-axenic strains. The observations reported here on new independently isolated axenic strains show that ω -amino acid sensitivity and axenic growth are not correlated; moreover, it is questionable whether strains AX2 and AX3 now in existence are in fact independently derived strains. Previous studies (Williams *et al.*, 1974*a, b*) and results reported here show that AX2 and AX3 share mutations, whose phenotype is indistinguishable but different from the parent NC4, at four loci (*axeA*, *axeB*, *axeC*, *oaaA*). Strains AX2 and AX3 share other characteristics which differ from the parent NC4, e.g. they are both partially temperature-sensitive for development when allowed to fruit on SM agar plates. The considerable similarity between AX2 and AX3 suggests that they are very closely related.

The genetic basis of axenicity has been examined more closely here than in previous studies and a new locus, *axeC*, whose phenotype is a doubling time of less than 15 h in axenic medium (when *axeA* and *axeB* are also present), is described on linkage group II. While sensitivity to ω -amino acids in AX2 and AX3 probably involves more than one locus (North & Ashworth, 1976), we have confirmed that the major determinant, which we have denoted *oaaA*, is on linkage group II. Analysis of haploids recombinant for linkage group II showed that *oaaA* and *axeA* are not allelic, which supports the view that ω -amino acid sensitivity and axenic growth are genetically different. However, as yet we have not been able to separate *axeC* and *oaaA*. Further studies are needed to determine whether or not reduced doubling time in axenic medium (*axeC*) and ω -amino acid sensitivity (*oaaA*) are pleiotropic phenotypes of the same locus.

Although the experiments reported here could not distinguish between tight binding and uptake, the concentration dependence of [^{14}C]EACA labelling suggests that a large proportion of cell-associated EACA does enter the cell. The uptake of EACA by *D. discoideum* is unlike that observed with rabbit retina which can accumulate EACA at concentrations above that in the medium (Ehinger, 1976), but is similar to the uptake of other amino acids by *D. discoideum* (Lee, 1972; M. J. North, unpublished). While there was some cell-associated EACA in resistant strains, sensitive strains showed much greater labelling. It seems most likely that sensitivity to EACA results from increased permeability to ω -amino acids. A possibility that we are investigating is that *oaaA* and *axeC* are, in fact, the same locus. If this was so, the decreased doubling time observed in axenic medium (*axeC*) could be explicable in terms of increased permeability to nutrients similar to ω -amino acids, possibly essential amino acids. Preliminary results suggest that the rate of leucine uptake is slower in strain NP82 than in strain AX2 (M. J. North, unpublished). There have been few investiga-

tions of the uptake of compounds by *D. discoideum* and no previous study comparing axenic and non-axenic strains. Studies with EACA have an advantage over those with nutrient compounds in that EACA is not metabolized. Further investigation may allow the molecular basis for increased permeability to be understood.

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