

Gene switching in *Amoeba proteus* caused by endosymbiotic bacteria

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Summary

The expression of genes for S-adenosylmethionine synthetase (SAMS), which catalyzes the synthesis of S-adenosylmethionine (AdoMet), a major methyl donor in cells, was studied in symbiont-free (D) and symbiont-bearing (xD) amoeba strains to determine the effect of bacterial endosymbionts. The symbionts suppressed the expression of the gene in host xD amoebae, but amoebae still exhibited about half the enzyme activity found in symbiont-free D amoebae. The study was aimed at elucidating mechanisms of the suppression of the amoeba's gene and determining the alternative source for the gene product. Unexpectedly, we found a second *sams* (*sams2*) gene in amoebae, which encoded 390 amino acids. Results of experiments measuring SAMS activities and amounts of

AdoMet in D and xD amoebae showed that the half SAMS activity found in xD amoebae came from the amoeba's SAMS2 and not from their endosymbionts. The expression of amoeba *sams* genes was switched from *sams1* to *sams2* as a result of infection with X-bacteria, raising the possibility that the switch in the expression of *sams* genes by bacteria plays a role in the development of symbiosis and the host-pathogen interactions. This is the first report showing such a switch in the expression of host *sams* genes by infecting bacteria.

Key words: Amoeba, Symbiosis, S-adenosylmethionine (AdoMet), S-adenosylmethionine synthetase (SAMS), Gene switching, Endosymbionts

Introduction

The xD strain of *Amoeba proteus* arose from the D strain by spontaneous infection of X-bacteria (Jeon and Lorch, 1967), and xD amoebae are now dependent on their symbionts for survival. Each xD amoeba contains about 42,000 symbionts within symbiosomes, and established xD amoebae die if their symbionts are removed. Newly infected xD amoebae become dependent on X-bacteria within 18 months (about 200 cell generations) (Jeon and Ahn, 1978), but the mechanism for the development of host dependence on their symbionts remains unknown. X-bacteria resemble *Legionella* sp. in their nucleotide sequences of *GroEL* genes (Ahn et al., 1994) and those of rRNA genes (K. J. Kim, Johns Hopkins University School of Medicine, Baltimore and K. W. Jeon, unpublished).

Earlier, we had found that symbiont-bearing xD amoebae did not transcribe the *sams* gene (*sams1*) encoding S-adenosylmethionine synthetase (SAMS) and no longer produced their own SAMS enzyme (Ahn and Jeon, 1983; Choi et al., 1997; Jeon and Jeon, 2003). However, xD amoebae still showed about half the level of SAMS activity found in symbiont-free D amoebae, despite their inability to express *sams*. Thus, it appeared that symbiotic X-bacteria suppressed the expression of the amoeba's *sams* and in turn provided the enzyme for their hosts, enforcing host amoebae to become dependent on symbionts themselves.

The SAMS enzyme catalyzes the formation of S-adenosylmethionine (AdoMet) from methionine and ATP (Thomas and Surdin-Kerjan, 1997) and is essential for cellular survival. AdoMet is the major methyl donor and precursor

for the biosynthesis of polyamines, spermidine and the phytohormone ethylene. In addition, it is an intracellular signal that controls essential cellular functions such as cell growth and differentiation in both eukaryotic and prokaryotic organisms (Kim et al., 2003; Mato et al., 2002; Shen et al., 2002; Thomas and Surdin-Kerjan, 1997). Therefore, lack of SAMS would be detrimental to cells.

In this study, we set out to determine if symbionts supplied SAMS or AdoMet to the host xD amoebae after inactivating the amoeba's own AdoMet production, by measuring SAMS activities in D and xD amoebae. We found that the expression of the normal amoeba *sams* gene (*sams1*) was switched to that of *sams2* by endosymbiotic X-bacteria. We present complete nucleotide and amino acid sequences of the second amoeba *sams* gene (*sams2*) and its product. Results of our study show that the SAMS activity detected in the xD amoeba cytosol comes from an amoeba SAMS isoform and not from the symbiont. This is the first report on switching of *sams* genes brought about by endosymbionts.

Materials and Methods

Amoebae

The D and xD strains of *A. proteus* were cultured in a modified Chalkley's solution (Jeon and Jeon, 1975) in Pyrex baking dishes (35×22×4 cm). Amoebae were fed every other day with axenically cultured and washed *Tetrahymena pyriformis* (Goldstein and Ko, 1976). The growth rates of amoebae were determined by growing individual amoebae in Syracuse watch glasses in Chalkley's medium

containing different concentrations of AdoMet and by counting the number of amoebae at intervals. AdoMet, in a stable form of sulfate-p-toluenesulfonate salt produced by Delta Pharmaceutical (Milan, Italy), was kindly provided by Dr Giovanni Frare. The culture media and food organisms were renewed every other day, and control groups were kept in Chalkley's solution without AdoMet. When desired, we removed X-bacteria from xD amoebae by growing them at 27°C (Jeon and Ahn, 1978).

Uptake of AdoMet by amoebae

Uptake of AdoMet by D and xD amoebae was measured using S-adenosyl-L-[methyl-³H]methionine (0.55 mCi/ml, 66.8 Ci/mmol) (³H] AdoMet) purchased from ICN Biomedicals (Irvine, CA). Amoebae were placed in Chalkley's solution at a density of 2×10³ cells/ml, and [³H] AdoMet was added to a final concentration of 2 μCi/ml. After adding [³H] AdoMet into the medium, 1 ml of amoebae was transferred to a 1.5 ml microcentrifuge tube at intervals and centrifuged for 1 minute at 2,000 g. The pelleted amoebae were used to measure the total amount of AdoMet taken up by cells, and an aliquot of the supernatant was used to calculate the AdoMet remaining in the medium. Amoebae were resuspended in 150 μl of 0.5 M HClO₄ and placed on 2.5-cm Whatman P-81 phosphocellulose paper discs (Choi et al., 1997). The radioactivity was measured in a Beckman scintillation spectrometer.

The amounts of AdoMet in the cytosol and AdoMet incorporated into macromolecules were estimated by measuring the total and incorporated AdoMet in soluble and insoluble fractions after sonicating amoebae for 15 seconds in 150 μl of 0.5 M HClO₄ followed by centrifugation for 5 minutes at 12,000 g.

Preparation of protein extracts and assay of SAMS activities

We extracted whole-cell proteins of amoebae by sonicating them briefly in 3 vol of an extraction buffer [100 mM Tris-HCl, pH 8.0, 5 mM DTT, 50 μM phenylmethanesulfonyl fluoride (PMSF)] and by centrifuging for 10 minutes at 13,000 g. The amount of proteins in the supernatant was determined by the Bradford method (Bradford, 1976), and 50–100 μg of proteins were used in the assay for the SAMS activity as described previously (Choi et al., 1997). X-bacteria inside xD amoebae were not broken by brief sonication and were precipitated by subsequent centrifugation. Therefore, SAMS activity of the supernatant obtained from xD amoebae represented that of host amoebae alone not including the symbionts' SAMS.

Determination of AdoMet level

AdoMet levels were determined as described by Shapiro and Ehninger (Shapiro and Ehninger, 1966). Amoebae (50–100 mg) were deproteinized by intermittently shaking with 2 vol of 1.5 M HClO₄ for 1 hour. The extract was neutralized with 3 M KHCO₃ and centrifuged for 20 minutes at 10,000 g. The supernatant was incubated with 300 μl of Dowex 50W-X8 resin (equilibrated in 0.1 M NaCl) for 30 minutes with agitation. The resin was washed with 0.1 M NaCl until OD₂₆₀ was below 0.05. AdoMet was eluted from the resin with 5 ml of 6 N H₂SO₄, and its concentration was determined by measuring the OD₂₅₆ (ε=15,400). The amount of AdoMet in xD amoebae represented the total AdoMet including that of X-bacteria in xD amoebae, since X-bacteria were completely disrupted by treatment with 1.5 M HClO₄ for 1 hour during AdoMet extraction. The amount of X-bacteria AdoMet was measured in X-bacteria isolated from xD amoebae. X-bacteria were isolated by a modification of the method of Kim et al. (Kim et al., 1994). In brief, xD amoebae were suspended in 5 vol of 20 mM Tris buffer (pH 7.4) by vigorous vortexing, sonicated for 10 seconds, and centrifuged for 5 minutes at 170 g. The pellet was resuspended in the same buffer, sonicated again, and centrifuged for 5 minutes at 170 g. The first and second supernatant

fractions were combined and centrifuged for 5 minutes at 7,000 g. The pellet was used to measure AdoMet level in the X-bacteria, after washing with the same buffer.

Fractionation of SAMS by Sephadex G-150 and DEAE-cellulose chromatography

SAMS was obtained by chromatography performed according to the method of Liau et al. (Liau et al., 1977). Cytosol extracts of amoebae were prepared by homogenizing them in 3 vol of 10 mM Tris-HCl (pH 7), 5 mM MgCl₂, 0.2 mM CaCl₂, and 10 mM DTT in a glass homogenizer. The resulting suspension was successively centrifuged for 10 minutes at 900 g and for 1 hour at 226,000 g. The cytosol extract (3 ml) was applied to a column (2.7×95 cm) of Sephadex G-150 equilibrated with 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 5 mM MgCl₂ and 20% glycerol. The same solution was used to elute the enzyme at a flow rate of 2.7 ml/fraction/15 minutes. The OD₂₈₀ profile was obtained for each fraction, and the SAMS profile was determined from a 25 μl aliquot from each fraction assayed for the enzyme activity. The Sephadex G-150 column was first calibrated with the following molecular markers; catalase (232 kDa), aldolase (158 kDa) and albumin (67 kDa). Blue dextran was used to determine the void volume (V₀).

DEAE-cellulose was washed with 2 M NaCl and water, and it was suspended in the same buffer used in Sephadex G-150 chromatography. The cytosol extract (3 ml) was applied to a DEAE-cellulose column (2.7×8 cm), and the column was washed with the same solution until unadsorbed proteins were washed off the column. The enzyme was then eluted with 150 ml of a linear gradient of 50 mM KCl/5 mM MgCl₂ to 500 mM KCl/50 mM MgCl₂ in 50 mM Tris-HCl (pH 7.8) and 20% glycerol. The OD₂₈₀ and SAMS profiles were obtained as described above. Active fractions were analyzed by western blotting using pAbs against amoeba SAMS1 (Jeon and Jeon, 2003).

Cloning the amoeba *sams2* gene

On the basis of a homology alignment of several amino acid sequences of SAMS (Choi et al., 1997; Jeon and Jeon, 2003; Yocum et al., 1996), we designed a pair of degenerate PCR primers corresponding to highly conserved amino acid sequences of known SAMS homologs. The 5' primer corresponded to nucleotides for amino acids 127–132 of amoeba SAMS1, Gly-Ala-Gly-Asp-Gln-Gly (5'-CCCGGATCC-GGNGCNGGNGAYCARGG-3'), and the 3' primer was for residues 308–314, Gly-Ala-Phe-Ser-Gly-Lys-Asp (5'-CCCGAATTCRTCYTT-NCYNGARAANBHNCC-3'), of the same SAMS (Jeon and Jeon, 2003). The primers introduced a *Bam*HI site at the 5' end and an *Eco*RI site at the 3' end of amplified fragments. PCR was performed under low-stringency annealing conditions (for 1 minute at 50°C with xD amoeba cDNA as the template). The cDNAs of xD amoebae were synthesized by reverse transcription with MMLV reverse transcriptase using oligo(dT) primers and mRNAs of xD amoebae, which had been extracted with the SV Total RNA Isolation System and PolyAtract mRNA Isolation System III (Promega). Amplified 500 bp fragments were cloned into pBSKII⁺ vectors and then sequenced. We obtained a 500 bp stretch of the amoeba *sams2* gene, which was different from the previously reported amoeba *sams1* (Choi et al., 1997), and used it to extend 5'- and 3'-end sequences.

The 5'- and 3'-end sequences of *sams2* were obtained by Rapid Amplification of cDNA Ends (RACE) (Frohman, 1993). For 3'-RACE analysis, cDNAs of xD amoebae, synthesized with oligo(dT) primers, were amplified with oligo(dT)-anchor primers (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTT-3') and *sams2*-specific forward primers (SAM1F) corresponding to nucleotides 435–452. First-round PCR products were reamplified with the adapter primers (5'-GACTCGAGTCGACATCG-3') and SAM2F primers, located at nt 715–732. Second-round PCR products were subcloned into a

pGEM-T Easy Vector (Promega) and sequenced. For 5'-RACE analysis, cDNAs were synthesized with a gene-specific SAM2R primer, followed by the attachment of poly(A) tails to cDNAs with dATP and terminal deoxynucleotidyl transferase (Promega). The tailed cDNAs were amplified with oligo(dT)-anchor primers and gene-specific SAM2R primers. First-round PCR products were reamplified with adapter primers and SAM1R primers. The SAM1R and 2R primers were located at the same positions as SAM1R and 2F primers, respectively, but in reverse sequences. Second-round PCR products were subcloned and sequenced.

Northern blot analysis

To determine the amounts of amoeba *sams1* and *sams2* transcripts, we introduced isolated X-bacteria into D-amoebae (Kim et al., 1994) and prepared total RNA using the SV Total RNA Isolation System (Promega) from samples collected every 4 days for 4 weeks after infection, as described previously (Jeon and Jeon, 2003). We carried out northern blot analysis by the usual method (Ausubel et al., 1992). For probing amoeba *sams1* and *sams2* mRNAs on northern blots, 1.1 kb fragments (nt 106-1,212) of *sams1* and 0.9 kb fragments (nt 1-910) of *sams2* amplified by PCR were labeled with [³²P]dCTP by the Prime-a-Gene labeling system (Promega). For probing *myosin* mRNA used as a control, 0.5 kb fragments (nt 989-1,455) of amoeba *myosin* (Oh and Jeon, 1998) were amplified and labeled with [³²P]dCTP to be used as probes.

Results

Growth rates of amoebae with added AdoMet

In order to test the hypothesis that the dependence of the xD amoeba on its symbionts for survival was related to the compensation of SAMS or AdoMet by the symbionts, we first checked growth rates of D and xD amoebae in media supplemented with AdoMet, following the removal of X-bacteria by growing xD amoebae for 12 days at 27°C. At room temperature, the growth patterns of both D and xD amoebae cultured in AdoMet concentrations of 10 and 50 µM were similar (Fig. 1). However, at 100 µM, D amoebae died within 10 days, and xD amoebae within 4 days. The results indicated that AdoMet at 100 µM was harmful to amoebae and that xD amoebae were more sensitive to a high concentration of AdoMet than were D amoebae. Results of our preliminary study showed that the expression of *sams1* in amoebae was down-regulated by AdoMet or Met treatment but there was no change in the expression of *sams2* (data not shown).

At 27°C all xD amoebae died within 2 weeks as was previously known. In contrast, D amoebae grew well even in 10 µM AdoMet but died after 11 days at concentrations of 50 and 100 µM AdoMet. The results indicated that D amoebae became sensitive to high temperature in 50 µM AdoMet, or higher, and that xD amoebae could not survive after the removal of X-bacteria even when AdoMet was supplied in the culture medium. However, the results did not exclude the possibility that X-bacteria provided SAMS or AdoMet to their host amoebae, since even D amoebae became sensitive to the high temperature in the presence of AdoMet.

Uptake of AdoMet into amoebae

Both D and xD amoebae showed the same pattern in time-dependent uptake of labeled AdoMet, but xD amoebae took up much more AdoMet than did D amoebae, the latter importing only 36% of the amount of AdoMet taken up by xD amoebae (Fig. 2). We checked the ratio between the amount of AdoMet in the cytosol and that of AdoMet incorporated into precipitable cellular components. For this purpose, we briefly sonicated radiolabeled amoebae in 0.5 M HClO₄ and separated them into insoluble precipitates and supernatants by centrifugation. Of the total AdoMet taken up by cells, 75% was found in the precipitates and 25% in supernatants both in D and xD amoebae. Both the total amount of AdoMet taken up and that incorporated into precipitable cellular components increased linearly, but the amount of AdoMet in the cytosol leveled off after the first 20 minutes. Since it was known that methylation of plasma-membrane phospholipids occurs without intracellular uptake of AdoMet (Bontemps and Van Den Berghe, 1997), we did not determine to which precipitable components the radioactivity was bound in this study.

Activity of SAMS

In order to determine if X-bacteria produced SAMS for their hosts, we measured SAMS activities of D and xD amoebae. xD Amoebae grown at room temperature had about 57% of the SAMS activity found in D amoebae. We also measured SAMS activities in symbiont-deprived xD amoebae grown for 8 days at 27°C to exclude any potential contamination with X-bacterial SAMS. Even though X-bacteria had been completely removed from xD amoebae by growing at 27°C, as checked under a phase-contrast microscope, xD amoebae showed the

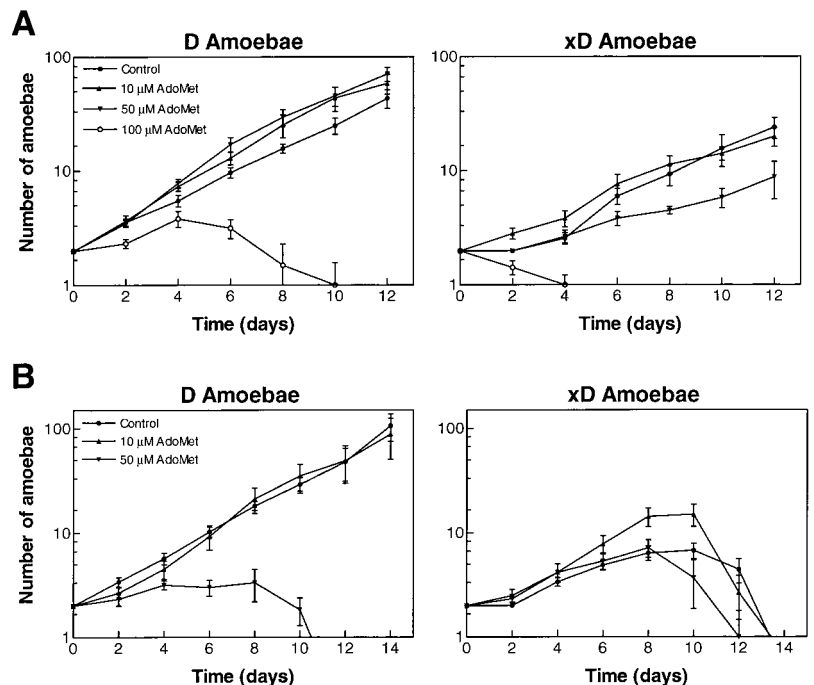


Fig. 1. Growth rates of amoebae at different concentrations of AdoMet. (A) Growth rates of D and xD amoebae at 22°C; (B) growth rates at 27°C. Each point represents the average (\pm s.d.) from 24 cells grown singly in two different experiments.

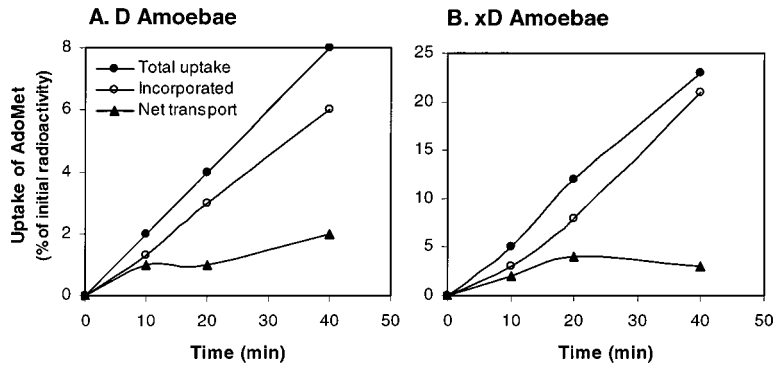


Fig. 2. Graphs to show the uptake of radiolabeled AdoMet (S-adenosyl-L-[methyl- 3 H]methionine) by D amoebae (A) and xD amoebae (B). S-Adenosyl-L-[methyl- 3 H]methionine was added to 2.0×10^3 amoebae/ml in Chalkley's solution to a final concentration of $2 \mu\text{Ci/ml}$. Total uptake (\bullet) was determined from collected amoebae before sonication. After sonication and centrifugation, incorporated AdoMet into macromolecules (\circ) was estimated from the pellets, and net transport (\blacktriangle) from supernatants. Results are expressed as percentage of the initial radioactivity of AdoMet. Values are averages from two separate experiments.

same level of SAMS activities as at room temperature (Fig. 3). The results indicated that the SAMS activity found in xD amoebae, which was about half that of D amoebae, came from host amoebae and not from X-bacteria. These results also indicated that the dependence of xD amoebae upon X-bacteria was SAMS-independent.

Characterization of SAMS from D and xD amoebae

The above results suggested that there might be a SAMS isoform in amoebae, with a different amino acid sequence from that of the previously studied amoeba SAMS (SAMS1). In order to check if there was such an isoform, we fractionated proteins of D and xD amoebae in Sephadex G-150 and DEAE-cellulose columns and identified SAMS by western blotting, using pAb against amoeba SAMS1. The elution patterns of SAMS from D and xD amoebae on Sephadex G-150 are shown in Fig. 4A. Three peaks with SAMS activity were detected in filtrates of the D amoeba cytosol extract, the regions corresponding to molecular masses of 210, 160 and 100 kDa, respectively. In contrast, most xD SAMS was eluted in two regions of 210 and 100 kDa, respectively.

Fig. 4B illustrates the elution patterns of SAMS from D and xD amoebae in DEAE-cellulose columns, showing enzyme profiles of D and xD amoebae to be different. While xD

amoeba SAMS was eluted as a sharp single peak at 0.2 M KCl, D amoeba SAMS was eluted as a broad major peak between 0.2 and 0.25 M KCl with a shoulder extending to 0.5 M KCl.

In order to check if amoeba SAMS1, the enzyme previously found only in D amoebae, was contained in the regions with peak enzyme activities, we analyzed aliquots of the fractions around the peak activity shown in Fig. 4A by SDS-PAGE and immunoblotting with pAb against amoeba SAMS1. Western blots showed a single band of 45 kDa in all fractions when aliquots of D amoebae were analyzed (Fig. 5). In contrast, no band was detected with the same pAb when aliquots of xD amoeba proteins were analyzed. When aliquots obtained by DEAE-cellulose chromatography were analyzed by western blotting, same results were obtained (data not shown). These results indicated that the SAMS activity detected in xD amoebal proteins did not come from amoeba SAMS1 and that there might be another amoeba SAMS (SAMS2) that was different from the previously reported SAMS1.

Complete nucleotide and deduced amino acid sequences of *sams2*

In order to clone the second *sams* gene (*sams2*) from amoebae, we reverse-transcribed and amplified mRNAs of xD amoebae. We obtained a 1.2 kb fragment of *sams2* by extending both ends of a 500 bp stretch of *sams2*, that had come from an amplified xD amoebal cDNA. We used degenerate PCR primers corresponding to DNA sequences encoding amino acids that were highly conserved among known SAMS (Choi et al., 1997; Yocum et al., 1996).

The *sams2* gene had an ORF of 1,173 nt (Fig. 6), encoding 390 amino acids, and it was similar to that of other organisms. The SAMS2 protein had a mass of 43 kDa, with pI 6.1, as determined by ExpASy Molecular Biology server.

Comparison of deduced amino acid sequences of SAMS2 with SAMS of other organisms

Multiple sequence alignment of amoeba SAMS2 with other SAMS homologs (Fig. 7) revealed that SAMS2 contained a consensus ATP-binding motif (GAGDQG at position 124-129; [compare Takusagawa et al. (Takusagawa et al., 1996)], the glycine-rich nanopeptide (GGGAFSGKD at position 271-279) (Choi et al., 1997; Jeon and Jeon, 2003), and metal-binding sites (Asp-24, Asp-285, and Glu-50) (see also McQueney and Markham, 1995; Reguera et al., 2002). Both X-bacteria SAMS and amoeba SAMS2 had a glycine residue at position 121,

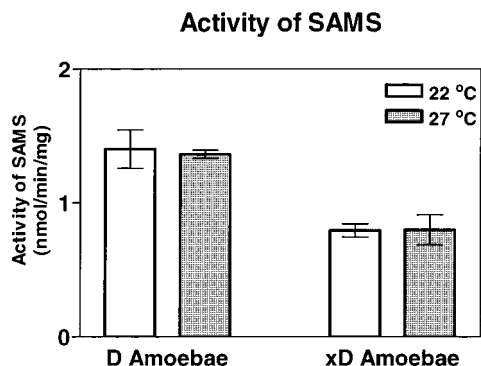


Fig. 3. Graphs to show SAMS activities in whole-cell proteins of D and xD amoebae as measured using L-[methyl- 3 H]methionine. To remove X-bacteria from xD amoebae, cells were grown for 8 days at 27°C. The values for xD amoebae represent only the SAMS activities of the host amoebae, not including that of the symbionts, since X-bacteria were excluded during extraction of proteins from xD amoebae. The data represent mean \pm s.d. from three separate experiments.

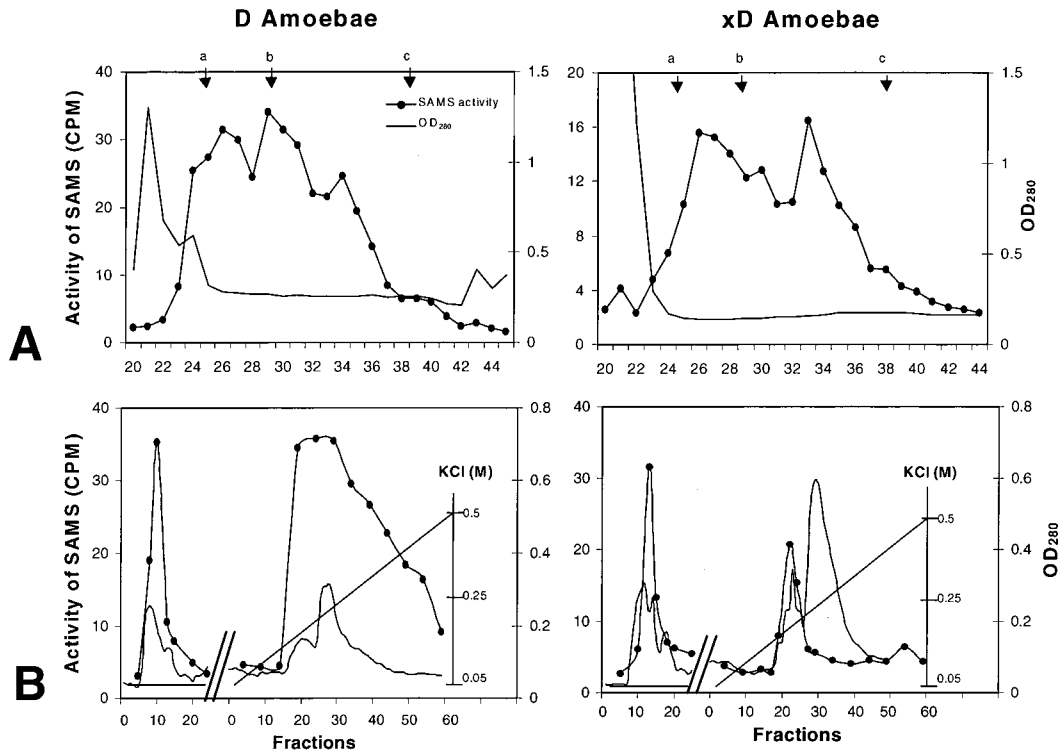


Fig. 4. Fractionation of SAMS from D and xD amoebae. (A) Results of Sephadex G-150 chromatography of D and xD amoebae. (B) Results of DEAE-cellulose chromatography of D and xD amoebae. Cytosolic extracts (2-3 ml) of D and xD amoebae were chromatographed, and 25 μ l aliquot from each fraction was used in the assay of SAMS activity. The Sephadex G-150 column was previously calibrated with the following molecular markers (arrows): a, catalase (232 kDa); b, aldolase (158 kDa) and c, albumin (67 kDa).

whereas SAMS1 had valine at the position. The sequence identity of SAMS2 with SAMS1 of *A. proteus* or that of X-bacteria was 50%, while the similarity with SAMS1 of *Acanthamoeba castellanii* was 72%, of *Phytophthora infestans* was 66% and of *E. coli* was 52%.

Northern blot analyses of *sams1* and *sams2* gene expression after infection

In order to ensure that the DNA sequence of the *sams2* gene was that of amoebae and not of X-bacteria, we analyzed genomic DNAs of D and xD amoebae and of X-bacteria by Southern blotting using a *sams2* probe, after digesting DNAs with *EcoRI*. We found a common band in DNAs of D and xD amoebae but not in X-bacteria DNA. In order to examine the expression profiles of *sams1* and *sams2* in newly infected xD amoebae, we infected D amoebae with X-bacteria and determined the amount of *sams* mRNA at intervals by northern blotting (Fig. 8). In agreement with our previous findings (Jeon and Jeon, 2003), the amount of *sams1* transcript decreased to a negligible level within 4 weeks following infection. In contrast, the amount of *sams2* transcript increased slightly 12-16 days after infection with X-bacteria. It appeared that the influence of X-bacteria on *sams* gene expression in amoebae was a rather slow process.

The amount of AdoMet

The above results suggested a possible switching between the

two *sams* genes in amoebae as a result of symbiosis with X-bacteria. To study the consequence of such switching, we measured the size of intracellular pools of AdoMet in the two amoeba strains and X-bacteria (Fig. 9). At room temperature, D amoebae contained 0.614 nmol/mg of AdoMet. xD amoebae had a similar amount of AdoMet (0.621 nmol/mg). This was calculated by subtracting the amount of X-bacterial AdoMet from that of xD amoebae containing X-bacteria. In order to check if X-bacteria provided AdoMet for their host amoebae without contributing the SAMS enzyme, we measured the AdoMet level after removing X-bacteria by growing xD amoebae for 8 days at 27°C. Even after the X-bacteria in xD amoebae were removed, levels of AdoMet in D and xD amoebae remained the same, indicating that X-bacteria did not provide AdoMet for their hosts.

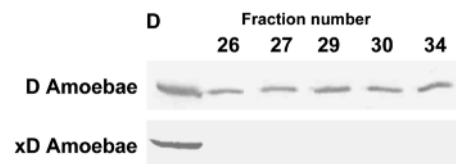


Fig. 5. Results of western blot analyses of proteins fractionated by Sephadex G-150 chromatography. Fractions containing SAMS enzyme activities (Fig. 4) were subjected to western blotting using pAb against amoeba SAMS1. A 15 μ l aliquot from each fraction was separated by SDS-PAGE (10%), and transferred to a membrane for western blotting. Whole-cell proteins of D amoebae were loaded in the first lane as a size marker for amoeba SAMS1.

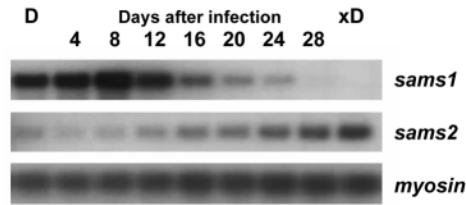


Fig. 8. Northern blot analyses of *sams1* and *sams2* genes following infection of D amoebae with X-bacteria. Total RNAs (20 µg) isolated from amoebae collected every 4 days after infection were separated in formamide gels and then transferred to nylon membranes. The signal was detected subsequently on the same blot with ^{32}P -labeled *sams1*, *sams2* and *myosin* probes. The *myosin* probe was used as a loading control.

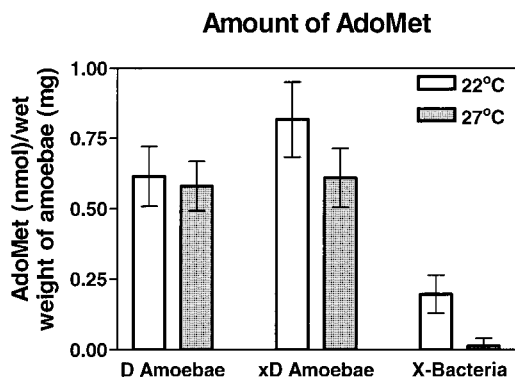


Fig. 9. Graphs to show the size of intracellular pools of AdoMet in amoebae and X-bacteria. Graphs for xD amoebae include the AdoMet of the symbiotic X-bacteria, as measured by using a Dowex 50W-X8 resin. The data represent mean \pm s.d. from three separate experiments.

growth stage (Thomas and Surdin-Kerjan, 1991), the expression of *sams2* being dependent upon the growth stage whereas that of *sams1* remains constant during growth. Liver cells expressing *MAT2A* have lower AdoMet levels than do cells expressing *MAT1A* (Cai et al., 1998). AdoMet is known to be a key molecule that differentially regulates *MAT1A* and *MAT2A* expression and it helps to maintain the differentiated status of the hepatocyte (Garcia-Trevijano et al., 2000). It has been suggested that fluctuations in the hepatic AdoMet concentration could be a part of the priming events and terminating signals that modulate the liver's regenerative process (Latasa et al., 2001). The hepatic AdoMet level is dramatically reduced shortly after a partial hepatectomy, and then subsequently it is restored to normal levels.

In our study, xD amoebae show a similar level of AdoMet as D amoebae. It is possible that the level of AdoMet fluctuates right after infection with X-bacteria as in the case of a partial hepatectomy. However, the effects of change in the amount of AdoMet in amoebae after infection with X-bacteria on the switch of *sams* gene expression and on the development of symbiosis between amoebae and X-bacteria remain to be examined.

In previous studies, we found that symbiont-bearing xD amoebae did not transcribe the *sams* gene and no longer produced their own SAMS as a result of harboring X-bacteria

(Ahn and Jeon, 1983; Choi et al., 1997; Jeon and Jeon, 2003). Yet, xD amoebae still showed about half the level of SAMS activity found in symbiont-free D amoebae. It appeared that X-bacteria suppressed the expression of the amoeba's *sams* and in turn provided the enzyme for their hosts. Thus, it was postulated that the lack of SAMS in xD amoebae was compensated by their bacterial endosymbionts and that such compensation might be the reason for amoebae becoming dependent on their symbionts for survival. Our present study was aimed at elucidating the mechanism for such gene suppression. Unexpectedly, our results show that the SAMS activity found in xD amoebae comes from the second amoeba SAMS (SAMS2) and not from endosymbionts. It appears that the reason why no SAMS was detected in xD amoebae in previous studies by immunoblotting using a pAb against amoeba SAMS1 was because SAMS2 of amoebae had only 50% identity in amino acid sequence with amoeba SAMS1 and thus it did not react with the pAb. Measurements of the SAMS activity and intracellular levels of AdoMet in amoebae show that X-bacteria do not provide SAMS or AdoMet for their hosts and that the dependence of xD amoebae on X-bacteria is AdoMet-independent. The intracellular level of xD amoeba AdoMet is similar to that of D amoebae even though xD amoebae possess only about half the SAMS activity of D amoebae.

Multiple sequence alignment of SAMS2 with other SAMS homologs reveals that SAMS2 also contains a consensus ATP-binding motif (GAGDQG) and the glycine-rich nanopeptide (GGGAFSGKD). The SAMS enzyme catalyzes the only known biosynthetic route to AdoMet, the primary biological methyl donor (McQueney et al., 2000). AdoMet also acts as an intracellular signal that controls essential cellular functions such as cell growth and differentiation as well as sensitivity to liver injury (Kim et al., 2003; Mato et al., 2002). In *Streptomyces*, an elevated level of AdoMet inhibits sporulation and a certain level of intracellular AdoMet is critical for the induction of antibiotic biosynthetic genes (Kim et al., 2003; Okamoto et al., 2003). In *E. coli*, a lowered level of AdoMet increases C to T mutagenesis (Macintyre et al., 2001) and results in a division defect (Newman et al., 1998). The administration of exogenous AdoMet attenuates liver injury induced by lipopolysaccharide treatment in rats (Chawla et al., 1998; Watson et al., 1999).

As shown by our study on the growth rates of amoebae, xD amoebae are more sensitive to exogenous AdoMet than are D amoebae. It is not known why xD amoebae are more sensitive to AdoMet, but it seems to be related to the fragility of the plasmalemma of xD amoebae. It is known that xD amoebae are more sensitive to overfeeding, starvation, microsurgical operations and elevated culture temperature (Jeon, 1995). Surprisingly, even D amoebae become sensitive to an elevated temperature in 50 µM or higher AdoMet. xD amoebae cannot survive at 27°C or above and all die within 2 weeks (Jeon and Ahn, 1978) because symbiotic bacteria disappear apparently by digestion (Lorch and Jeon, 1980). Our results show that amoebae themselves become temperature sensitive in the presence of AdoMet.

Enteric bacteria are not permeable to AdoMet (Sekowska et al., 2000), but *S. cerevisiae* is capable of actively transporting AdoMet (Thomas and Surdin-Kerjan, 1997). It is not clear if mammalian cells take up exogenous AdoMet and conflicting

data are found in the literature (Bontemps and Van Den Berghe, 1997; Watson et al., 1999). Meanwhile, intracellular levels of AdoMet in isolated rat hepatocytes increase when external AdoMet concentration is 200 μM or higher (Watson et al., 1999). Incubation of isolated rat hepatocytes with 2 or 50 μM AdoMet causes the methyl group of exogenous AdoMet to be incorporated, without intracellular uptake, into phospholipids most probably situated on the outside of the plasma membrane, forming phosphatidylcholine (Bontemps and Van Den Berghe, 1997).

It is interesting that amoebae are permeable to AdoMet in the medium, and that most of the exogenous AdoMet taken up is incorporated into precipitable cell components without concomitant intracellular uptake in the cytosol (cf. Fig. 2). Both D and xD amoebae showed time-dependent uptake of AdoMet, but xD amoebae took up much more exogenous AdoMet than did D amoebae. It is probable that the radiolabeled methyl group of AdoMet was incorporated into phospholipids of the plasma membrane as in other cases (Bontemps and Van Den Berghe, 1997). The temperature sensitivity of amoebae in the presence of AdoMet may be due to the change in the fluidity of amoeba membrane caused by exogenous AdoMet. The higher uptake rate of exogenous AdoMet by xD amoebae appears to be the reason for higher sensitivity of xD amoeba to AdoMet, since a high concentration of AdoMet is harmful to amoebae.

Genomic and cDNA clones of *sams* have been obtained for various organisms (Horikawa and Tsukada, 1991; Jeon and Jeon, 2003; Markham et al., 1984; Peleman et al., 1989; Sakata et al., 1993; Schroder et al., 1997; Thomas and Surdin-Kerjan, 1987). Most organisms studied to date have more than one SAMS isozymes. The *sams* genes are a small gene family, and they appear to be highly conserved, at least within a species (Mato et al., 2002; Reguera et al., 2002; Shen et al., 2002; Thomas and Surdin-Kerjan, 1997).

The amoeba SAMS2 enzyme we identified has only 50% amino acid sequence identity with SAMS1 of amoebae. The sequence identity between the two isoforms in amoebae is unusually low since all other known SAMS isoforms of an organism show over 80% sequence identity. Based on the sizes of proteins determined by gel filtration, there seem to be three different conformations of D amoeba SAMS, while there seem to be only two different conformations of SAMS in xD amoebae. However, the reason why amoeba SAMS has different sizes is not clear.

In amoebae, *sams2* is expressed at a low level in symbiont-free D amoebae but it is activated by X-bacteria. The amount of AdoMet in xD amoebae measured after the removal of X-bacteria is similar to that of D amoebae, suggesting that X-bacteria do not provide AdoMet for their host amoebae. It is interesting that xD amoebae show only half the activity of SAMS while containing a similar amount of AdoMet as D amoebae. The discrepancy between SAMS activity and the amount of AdoMet in xD amoebae might be due primarily to a lower estimate of SAMS activity in xD amoebae. Possible differences in K_m values for the enzyme substrates present in the two amoeba strains or different V_{max} of the enzymes might have caused lower estimation of SAMS activity in xD amoebae. In our study, we used constant concentrations of substrates, methionine and ATP for measuring the activity of SAMS. Different properties of SAMS isozymes have been

reported in mammals (Mato et al., 2002) and in yeast (Thomas and Surdin-Kerjan, 1997).

Further work is in progress to determine the mechanism and consequences of the *sams* gene switching in amoeba/X-bacteria symbiosis. The switch in gene expression in amoebae is not only an example of genetic alterations caused by host-symbiont interactions but also may serve as a good model to study interactions between hosts and infective agents such as *Mycobacterium*, *Legionella*, *Toxoplasma*, *Salmonella*, and others.

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