contacted by the appropriate upstream kinase (MKK3 in this instance) and directing it to phosphorylate the residues in the T-loop. Indeed, the αC helix of Cdk2 interacts with the regulatory subunit cyclin A, reorienting the T-loop and opening the entrance of the catalytic cleft (*17*). The αC helix might therefore represent a key exposed region used by protein kinases to receive input regulatory signals.

By generating chimeric MAPK able to convert stress signals into growth factor responses, we have demonstrated that signal reception domains may be dissociable from signal delivery domains. If this model can be generalized to other members of the MAP kinase family as well as to the upstream kinases of the transduction cascades, a new class of specific "MAPK module" antagonists could be created by targeting domains specifying agonist activation.

REFERENCES AND NOTES

1. A. M. Neiman, *Trends Genet.* **9**, 390 (1993).
2. T. W. Sturgill and J. Wu, *Biochem. Biophys. Acta* **1092**, 350 (1991); T. G. Boulton *et al.*, *Cell* **65**, 663 (1991).
3. J. Han, J.-D. Lee, L. Bibbs, R. J. Ulevitch, *Science* **265**, 808 (1994); N. W. Freshney *et al.*, *Cell* **78**, 1039 (1994); J. C. Lee *et al.*, *Nature* **372**, 739 (1994).
4. J. Rouse *et al.*, *Cell* **78**, 1027 (1994).
5. B. Dérijard *et al.*, *ibid.* **76**, 1025 (1994); J. M. Kyriakis *et al.*, *Nature* **369**, 156 (1994).
6. G. Pagès *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8319 (1993); S. Cowley, H. Paterson, P. Kemp, C. J. Marshall, *Cell* **77**, 841 (1994); S. J. Mansour *et al.*, *Science* **265**, 966 (1994); A. Brunet, G. Pagès, J. Pouysségur, *Oncogene* **9**, 3379 (1994).
7. B. Dérijard *et al.*, *Science* **267**, 682 (1995); I. Sanchez *et al.*, *Nature* **372**, 794 (1994).
8. A. Brunet and J. M. Brondello, unpublished results.
9. D. O. Morgan and H. L. De Bondt, *Curr. Opin. Cell Biol.* **6**, 239 (1994).
10. E. J. Goldsmith and M. H. Cobb, *Curr. Opin. Struct. Biol.* **4**, 833 (1994).
11. S. K. Hanks, A. M. Quinn, T. Hunter, *Science* **241**, 42 (1988).
12. F. Zhang, A. Strand, D. Robbins, M. H. Cobb, E. J. Goldsmith, *Nature* **367**, 704 (1994).
13. T. H. Chen, Sarnecki, C. J. Blenis, *Mol. Cell. Biol.* **12**, 915 (1992); D. Stokoe *et al.*, *EMBO J.* **11**, 3985 (1992).
14. R. Treisman, *Curr. Opin. Genet. Dev.* **4**, 96 (1994).
15. C. Albanese *et al.*, *J. Biol. Chem.* **270**, 23589 (1995); J. Lavoie, N. Rivard, G. L'Allemain, J. Pouysségur, *Progress in Cell Cycle Research*, L. Meijer and S. Guidet, Eds. (Plenum, New York, 1996), vol. 2, in press.
16. A. Brunet and J. Pouysséqur, unpublished results.
17. P. D. Jeffrey *et al.*, *Nature* **376**, 313 (1995).
18. S. Meloche, G. Pagès, J. Pouysségur, *Mol. Biol. Cell* **3**, 63 (1992).
19. p44MAPK was NH_2-terminally tagged with the HA epitope (MYDVPDYASLP) replacing the first 10 codons and subcloned in the pECE expression vector driven by an SV40 promoter. Murine p38MAPK complementary DNA (cDNA) was NH_2-terminally tagged at the first codon with the HA epitope and subcloned in the pECE vector. The 293 cells (6×10^5 cells per 35-mm well) were transfected with 6 μg of DNA by the calcium phosphate technique. Two days after transfection, cells were serum-deprived for 5 hours and stimulated with various agonists. Kinase assays were done as described (*18*). Briefly, cells were lysed in 400 μl of Triton lysis buffer. Equal amounts of proteins (300 μg) were immunoprecipitated on protein A–Sepharose beads coupled with the antibody to HA (anti-HA) (Babco, Emeryville). Activity of the kinases were assayed in 40 μl of kinase buffer with various substrates: glutathione-S-transferase (GST)–ATF2 (1–109), GST-Myc (1–143), GST-Elk1 (307–428), and MBP at a final concentration of 10 μg and 3 μCi of [γ-^{32}P], 50 μM adenosine 5′-triphosphate (ATP) (ICN) for 30 min at 30°C. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS–polyacrylamide gel electrophoresis (PAGE); the gels were autoradiographed, stained with Coomassie brilliant blue, and the bands corresponding to the substrates were excised and counted.
20. All chimerae were constructed from p44 and p38 MAPK vectors by introduction of polymerase chain reaction (PCR) fragments through use of restriction sites in p38MAPK [Ava I [93 base pairs (bp), domain I], PpuM I (200 bp, domain III), BstE II (312 bp, domain V), Sac I (491 bp, domain VII), and Kpn I (564 bp, domain VIII)] or in p44MAPK [Afl II (508 bp, domain VII) and Kpn I (600 bp, domain VIII)]. All the PCR fragments and the junctions were verified by sequencing.
21. Protein immunoblot experiments were done with anti-HA after resolution of proteins on SDS-PAGE (*18*).
22. The 293 cells (6×10^5 cells per 35-mm well) were cotransfected with 0.2 μg of the plasmid encoding avian p90^{rsk} and 1 μg of the relevant constructs. Two days after transfection, cells were serum-deprived for 5 hours and stimulated by various agonists. Cells were lysed in the Triton lysis buffer, and equal amounts of proteins (300 μg) were immunoprecipitated with anti-HA. Kinase assays were done for 30 min at 30°C either with 10 μg of MBP, 3 μCi of [γ-^{32}P], 50 μM ATP to measure MAPK activity or with 3 μCi of [γ-^{32}P], 1.5 μM ATP to measure phosphorylation of associated p90^{rsk}. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS-PAGE (7.5%); the gel was autoradiographed, transferred onto nitrocellulose, and immunoblotted with an antibody directed against p90^{rsk} (Santa Cruz).
23. The 293 cells (6×10^5 cells per 35-mm well) were cotransfected with 3 μg of the plasmid encoding avian p90^{rsk} and 3 μg of the relevant constructs. Two days after transfection, cells were serum-deprived for 5 hours and stimulated by various agonists. Cells were lysed in the Triton lysis buffer, and equal amounts of proteins (300 μg) were immunoprecipitated with an antibody directed against avian p90^{rsk}. Kinase assays were done as described [R.-H. Chen and J. Blenis, *Mol. Cell. Biol.* **10**, 3204 (1990)] in 40 μl of kinase buffer, 20 μg of 40S subunit, and 3 μCi of [γ-^{32}P], 50 μM ATP for 15 min at 30°C. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS-PAGE and the gel was autoradiographed.
24. CCL39 cells (10^5 cells per well in 24-well culture plates) were transfected with 0.25 μg of the c-*fos*–luciferase reporter gene and 0.75 μg of the relevant constructs. One day after transfection, cells were incubated in serum-free medium for 24 hours, then stimulated with fetal calf serum (20%) or IL-1β (10 ng/ml; Boehringer) for 16 hours. Luciferase activity was measured according to the Promega protocol.

25. We thank J. Blenis for providing the plasmid encoding the avian p90^{rsk} and the antibody directed against it, R. Davis for the GST-ATF2 construct, D. Czernilofsky for the c-*fos* promoter–luciferase construct, R. Ulevitch for the p38MAPK cDNA, G. Thomas for the 40S ribosomal S6 protein and R. Treisman for the GST-elk1 construct, D. Grall and M. Valetti for technical assistance, B. Chabanne for assistance with photography, V. Dulić and F. R. McKenzie for helpful comments on the manuscript, and J.-C. Chambard, B. Dérijard, and G. Pagès for stimulating discussions. We are especially indebted to V. Dulić for help and encouragement. Supported by the CNRS, the Institut National de la Santé et de la Recherche Médicale/MSD 91AN13, the Ligue Nationale contre le Cancer, and the Association pour la Recherche contre le Cancer.

4 December 1995; accepted 10 April 1996

Enzymatic Synthesis of a Quorum-Sensing Autoinducer Through Use of Defined Substrates

Margret I. Moré, L. David Finger, Joel L. Stryker, Clay Fuqua, Anatol Eberhard, Stephen C. Winans*

Many bacteria, including several pathogens of plants and humans, use a pheromone called an autoinducer to regulate gene expression in a cell density–dependent manner. *Agrobacterium* autoinducer [AAI, *N*-(3-oxo-octanoyl)-L-homoserine lactone] of *A. tumefaciens* is synthesized by the TraI protein, which is encoded by the tumor-inducing plasmid. Purified hexahistidinyl-TraI (H_6-TraI) used *S*-adenosylmethionine to make the homoserine lactone moiety of AAI, but did not use related compounds. H_6-TraI used 3-oxo-octanoyl–acyl carrier protein to make the 3-oxo-octanoyl moiety of AAI, but did not use 3-oxo-octanoyl–coenzyme A. These results demonstrate the enzymatic synthesis of an autoinducer through the use of purified substrates.

Mechanisms that allow bacteria to control gene expression in a cell density–dependent manner have evolved independently a number of times (*1*, *2*). This type of gene expression is referred to as quorum-sensing. Probably the best characterized quorum-sensing system is the LuxR-LuxI system of *Vibrio fischeri*. LuxI protein produces *V. fischeri* autoinducer [VAI, *N*-(3-oxo-hexanoyl)-L-homoserine lactone], which binds to the transcriptional activator protein LuxR (*3*, *4*). Complexes of LuxR-VAI activate transcription of the *lux* operon, resulting in bioluminescence. Because VAI diffuses passively across the cell envelope, high intracellular concentrations of VAI are attained only in the presence of neighboring VAI-producing bacteria (*5*). Similar regulatory systems are found in a broad variety of eubacteria, many

M. I. Moré, J. L. Stryker, S. C. Winans, Section of Microbiology, Cornell University, Ithaca, NY 14853, USA.
L. D. Finger and A. Eberhard, Department of Chemistry, Ithaca College, Ithaca, NY 14850, USA.
C. Fuqua, Department of Biology, Trinity University, San Antonio, TX 78212, USA.
*To whom correspondence should be addressed.

of which are pathogens. Each of these systems uses an autoinducer that is composed of a homoserine lactone moiety linked to an acyl chain of variable length and oxidation state (*1*, *2*). Among these, the TraR and TraI proteins of *A. tumefaciens* (homologous to LuxR and LuxI, respectively) and the pheromone AAI [*N*-(3-oxo-octanoyl)-L-homoserine lactone] regulate the conjugal transfer genes of the tumor-inducing (Ti) plasmid as well as at least six genes not required for conjugation (*6*, *7*).

Expression of the related LuxI, TraI, or LasI proteins in *Escherichia coli* is sufficient for synthesis of the cognate autoinducers (*3*, *6*, *8*), an indication that these proteins provide substrate specificity. Before this study almost nothing was known about the substrates or reaction mechanisms of these proteins. It has been proposed that the conserved homoserine lactone moiety of the *Vibrio* autoinducer VAI is derived from *S*-adenosylmethionine (AdoMet) (*9*) or from homoserine lactone (*10*). It has been suggested that the diverse fatty acid moieties are derived from different intermediates in fatty acid biosynthesis (*11*), although they could also be derived from intermediates in fatty acid degradation.

To learn more about the reactions catalyzed by this family of proteins, we purified TraI and used it to reconstitute AAI synthesis in vitro. We fused the *traI* gene to six histidine codons, creating pJS101 (*12*). The *E. coli* strain BL21/DE3(pJS101) released large amounts of AAI into the culture supernatant (*13*), indicating that this fusion protein was active in vivo. Initial attempts to synthesize AAI in vitro with affinity-purified hexahistidinyl-TraI (H_6-TraI) (*12*) and an extract of soluble *E. coli* proteins (S28 extract) were unsuccessful. However, when AdoMet was combined with H_6-TraI and S28 extract, AAI was synthesized (Table 1). Synthesis required both H_6-TraI protein and S28 extract. We did not detect H_6-TraI–dependent AAI production when homoserine lactone was used in place of AdoMet.

We used gel filtration chromatography to determine the molecular mass of H_6-TraI under nondenaturing conditions (*14*). The sole peak of AAI-synthesizing activity eluted at 22,000 daltons. Because H_6-TraI has a molecular mass of 25,012 daltons, we conclude that this protein is monomeric.

To provide additional evidence that AdoMet is a direct precursor for AAI, we repeated the assays described above using *S*-adenosyl-L-[carboxyl-^{14}C]methionine and separated AAI from the reactants using reversed-phase high-performance liquid chromatography (HPLC). A peak of *tra* gene–stimulatory activity eluted between 4.25 and 4.5 min (Fig. 1A), which corresponds to the retention time of chemically synthesized AAI (Fig. 1D). This bioactivity precisely coeluted with a peak of radioactivity (Fig. 1B). Both the radioactivity (Fig. 1C) and the bioactivity (*15*) were absent in a control reaction lacking H_6-TraI. We conclude that AdoMet is a precursor of AAI.

To investigate the origin of the fatty acid moiety of AAI, we sought to identify low molecular weight molecules in the S28 extract that were required for activity. Dialysis of the extract abolished activity (Table 2). Activity was restored by the addition of malonyl–coenzyme A (CoA) and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), two substrates in fatty acid biosynthesis (*16*). We found that NADH (re-

Table 1. Requirement of *S*-adenosylmethionine for in vitro AAI synthesis. AAI synthesis reactions (70 μl) were carried out for 10 min at 22°C; each reaction contained 10 mM tris-HCl (pH 7.4), 330 mM NaCl, 15% glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$ and, unless otherwise indicated, 0.15 mM AdoMet (or homoserine lactone), *E. coli* S28 extract (final concentration, 3.5 mg of protein per milliliter), and 6 μg of H_6-TraI per milliliter. We prepared *E. coli* S28 extract by culturing strain BL21/DE3 in LB medium at 37°C to mid-log phase, resuspending cells in TEDG buffer [10 mM tris-HCl (pH 8), 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol] and disrupting them with a French Press minicell (20,000 pounds per square inch). The lysate was centrifuged for 1 hour at 28,000*g*, and the supernatant (~5 mg of protein per milliliter) was retained for AAI assays. We stopped the reactions by adding three volumes of ethyl acetate. The ethyl acetate phase was transferred and evaporated under vacuum at 60°C, and the residue was resuspended in defined AT growth medium (*25*) that had been inoculated with *A. tumefaciens* strain A136(pCF218) (pCF372). This strain lacks a Ti plasmid, contains a *traI-lacZ* fusion, and overproduces TraR (*26*). After overnight incubation, we measured the β-galactosidase specific activity using mid-log phase cultures. We obtained a standard dose-response curve, using synthetic racemic AAI. This assay was quantitative between 0.15 nM and 5.0 nM L-AAI.

Assay components	β-Galactosidase units	AAI production (pmol min^{-1} per microgram of H_6-TraI)
TraI, S28, AdoMet	394	21.9
S28, AdoMet	<1	<0.01
TraI, AdoMet	<1	<0.01
TraI, S28	<1	<0.01
TraI, S28, homoserine lactone	4	0.22
S28, homoserine lactone	4	0.22

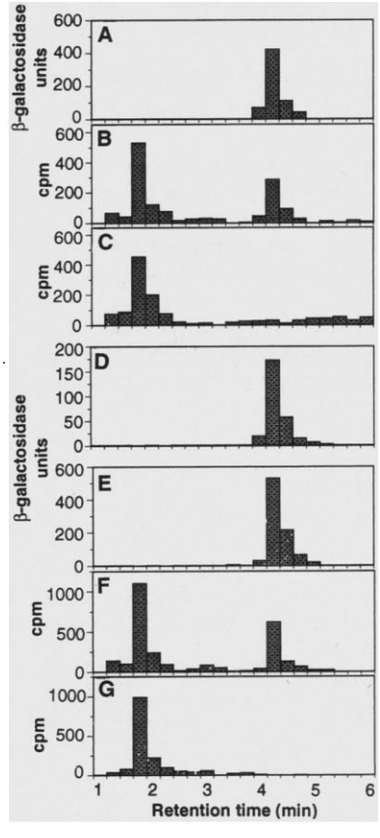

Fig. 1. Conversion of radiolabeled AdoMet to AAI by H_6-TraI. (**A**) Bioassay with S28 extract and H_6-TraI. (**B**) Radioactivity of each fraction of (A). (**C**) Radioactivity of fractions with S28 extract in the absence of H_6-TraI. (**D**) Synthetic nonradiolabeled AAI. (**E**) Bioassay of reactions carried out with Ado-Met, OOACP, and H_6-TraI in a defined buffer. (**F**) Radioactivity of fractions of (E). (**G**) Radioactivity of fractions from reactions in the presence of OOACP but lacking H_6-TraI. Reactions (A) through (C) contained 0.05 mM *S*-adenosyl-L-[carboxyl-^{14}C]methionine (Amersham), 2.7 mg of S28 extract per milliliter and 0.4 μg of H_6-TraI per milliliter in a buffer containing 330 mM NaCl, 15% glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$, and 40 mM tris-HCl (pH 7.4). Reactions (E) through (G) contained 0.11 mM *S*-adenosyl-L-[carboxyl-^{14}C]methionine, 7 μg of OOACP and 17 μg of ACP per milliliter, 1.1 μg of H_6-TraI per milliliter, in a buffer containing 100 mM NaCl, 5% glycerol, 1 mM DTT, 2 mM EDTA, and 65 mM tris-HCl (pH 7.4). Reactions were allowed to continue for 1 hour at 22°C and were terminated by extraction with ethyl acetate. Ethyl acetate was separated and evaporated. We resuspended the residue in a 50% methanol-water mixture and fractionated it by isocratic reversed-phase HPLC, using a polycyclic aromatic hydrocarbon Hypersil column (Keystone Scientific) and a 50% methanol-water mixture (1 ml min^{-1}). Fractions were evaporated and the residue was resuspended in water for AAI bioassays as described in Table 1 and for radioactivity with a Beckmann 5000CE scintillation counter. Background counts were subtracted.

duced form of nicotinamide adenine dinucleotide) was approximately as effective as NADPH in supporting in vitro AAI synthesis. Acetyl-CoA did not function in place of malonyl-CoA and did not stimulate reactions containing malonyl-CoA (*15*). To our knowledge, the only known reactions in *E. coli* that require malonyl-CoA are those that occur in fatty acid biosynthesis.

Further evidence that the acyl moiety of AAI was derived from fatty acid biosynthetic intermediates was provided by the use of cerulenin, an inhibitor of fatty acid biosynthesis (*16*). Cerulenin is an irreversible inhibitor of β-ketoacyl–acyl carrier protein (ACP) synthase I and II activities that acts by covalently binding to the fatty acyl binding sites of these enzymes (*17*). We treated dialyzed S28 extract with varying concentrations of cerulenin, removed cerulenin by dialysis, and assayed for AAI synthesis in the presence of H_6-TraI, AdoMet, malonyl-CoA, and NADPH. Synthesis of AAI was 50% inhibited by treatment with 6.3 μM cerulenin (Fig. 2, solid line), a concentration six times as great as that reported to cause 50% inhibition of β-ketoacyl–ACP synthase I (KAS I) (*18*). Treatment of H_6-TraI with cerulenin did not affect AAI synthesis (Fig. 2, dashed line). We conclude that AAI synthesis in these assays requires ongoing fatty acid biosynthesis.

Because nascent fatty acids are bound to ACP, we hypothesized that the 3-oxo-octanoyl moiety might be obtained directly from ACP. To test this hypothesis, we prepared 3-oxo-octanoyl-ACP using the reagent 3-oxo-octanoylthiocholine iodide (OOTC, Fig. 3A). The positive charge of the quaternary amine has been shown to facilitate the transfer of other acyl groups to the thiol group of CoA (*19*). Because ACP has a similar phosphopantetheine moiety (and no other thiol groups), we reasoned that OOTC might transfer its 3-oxo-octanoyl group to the thiol group of ACP. We therefore incubated OOTC with ACP, precipitated ACP with trichloroacetic acid (TCA) to stop the reaction and remove OOTC (*20*), and visualized ACP and acyl-ACP by native gel electrophoresis. Acyl-ACPs migrate slightly faster than ACP in these gels (*21*). Incubation of ACP with OOTC increased the mobility of ~50% of the ACP molecules (Fig. 3B), strongly suggesting that this reaction created 3-oxo-octanoyl-ACP (OOACP).

To determine whether OOACP is a substrate for AAI biosynthesis, we incubated OOACP with H_6-TraI, and radiolabeled AdoMet in a defined buffer, and fractionated the products by reversed-phase HPLC. Both the bioactive fraction and the radioactivity coeluted with chemically synthesized AAI (Fig. 1, E and F). Control reactions lacking H_6-TraI did not produce detectable AAI (Fig. 1G). In reactions containing H_6-TraI, OOACP, and AdoMet, the maximum rate of AAI synthesis was 1.03 mol min^{-1} per mol of H_6-TraI (*22*). The Michaelis constant (K_M) for AdoMet was 48 μM [regression coefficient (R^2) = 0.9924], and the K_M for OOACP was 0.33 μM (R^2 = 0.9807). The maximum V (V_{max}) of TraI may seem low compared with those of other enzymes and could be due to loss of activity during purification.

Table 2. Requirement of malonyl-CoA and NADPH for AAI production. AAI synthesis reactions (90 μl) were allowed to continue for 30 min at 22°C and contained 0.24 mM AdoMet, 1.1 μg of H_6-TraI per milliliter, 10 mM tris-HCl (pH 7.4), 330 mM NaCl, 15% glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, and 0.1 mM $FeSO_4$. Nondialyzed S28 extract (final concentration, 2.75 mg of protein per milliliter), dialyzed S28 extract (dS28) (final concentration, 2.75 mg of protein per milliliter), and 0.14 mM malonyl-CoA, 1.1 mM NADH, and 1.1 mM NADPH were added as indicated.

Assay components	β-Galactosidase specific activity	AAI production (pmol min^{-1} per microgram of H_6-TraI)
S28	159	19.3
dS28	<1	<0.01
dS28, malonyl-CoA, NADPH	117	12.0
dS28, malonyl-CoA, NADH	111	11.6
dS28, malonyl-CoA	<1	<0.01
dS28, NADPH	<1	<0.01

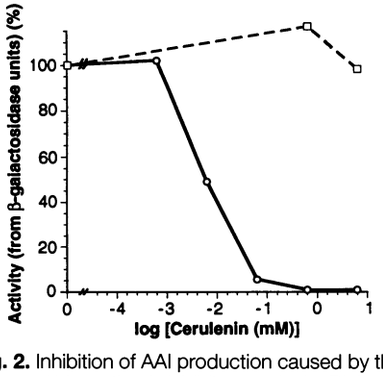

Fig. 2. Inhibition of AAI production caused by the fatty acid biosynthesis inhibitor cerulenin. Dialyzed S28 extract (solid line) or H_6-TraI (dashed line) were incubated with cerulenin (Sigma) at the concentrations indicated for 1 hour at 4°C, dialyzed overnight, and assayed for AAI production as described in Table 1.

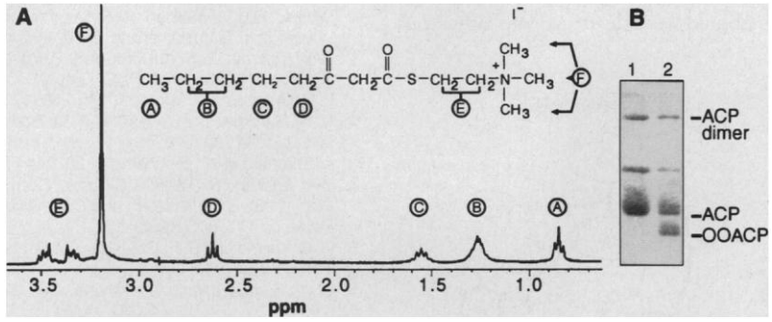

Fig. 3. Chemical acylation of ACP by OOTC. (**A**) Proton NMR of OOTC. To synthesize OOTC, we combined hexanoylchloride with Meldrum's acid in methylene chloride in the presence of pyridine at 0°C for 1 hour and then at 23°C for an additional hour. The resulting hexanoyl–Meldrum's acid was dried by rotoevaporation, characterized by proton NMR ($CDCl_3$) and Fourier transform infrared spectroscopy (FTIR), and resuspended in benzene. A mixture of 12 mmol of 2-dimethylaminoethanethiol and 10 mmol of hexanoyl–Meldrum's acid in 100 ml of dry benzene was refluxed for 3 hours and allowed to stand at 23°C for 1.5 hours. Benzene was removed by rotoevaporation, and the resulting red-orange oil was applied directly to a silica gel column (3 cm by 30 cm, 22 grade, 60 to 200 mesh) and eluted isocratically with diethylether-acetone (30:70 v/v). The eluate was dried by rotoevaporation. The result was an orange oil, which was characterized by proton and ^{13}C NMR ($CDCl_3$) and FTIR. The oil (7 mmol) was dissolved in diethylether-acetone (60:40 v/v), and 9 mmol of methyl iodide was added. After overnight incubation at 23°C, the solvents were rotoevaporated. The residue (OOTC) was characterized by proton NMR (D_2O). The presence of the 3-oxo group, the thioester, and the 2-methylene group was confirmed by FTIR and ^{13}C NMR (D_2O). A 0.5 M OOTC stock solution was prepared in acetonitrile. 2-Dimethylaminoethenethiol was purchased from Research Organics; all other reagents were purchased from Aldrich. (**B**) Conformationally sensitive polyacrylamide gel electrophoresis (PAGE) (*21*) of ACP (lane 1) and OOACP (lane 2). ACP (Sigma) was treated with DTT and hydroxylamine, precipitated with TCA, and washed as described. We performed the acylation reaction by incubating ACP (10 mg ml^{-1}) and 5 mM OOTC in a 40 mM phosphate buffer containing 25 mM NaCl, 5% glycerol, and 2 mM EDTA (pH 7.4). After 1 hour the reaction was stopped by precipitation with TCA. The precipitated ACP and ACP derivatives were washed as above and resuspended in the same buffer, but containing 50% glycerol.

Alternatively, a low V_{max} in vivo could be advantageous. Because *A. tumefaciens tra* genes can be induced by as low a concentration as 0.1 nM AAI (*6*, *7*), a higher rate of AAI synthesis could cause target gene induction at inappropriately low cell densities. Control reactions containing ACP in place of OOACP did not produce AAI.

To determine whether 3-oxo-octanoyl-CoA (OOCoA) can substitute for OOACP as a H_6-TraI substrate, we used OOTC to acylate CoA, purified OOCoA by reversed-phase HPLC, and verified its structure by nuclear magnetic resonance (NMR) spectroscopy (*23*). Reactions containing OOCoA, H_6-TraI, and AdoMet but lacking OOACP did not produce detectable AAI (*15*). We also tested whether compounds other than AdoMet could provide the homoserine lactone moiety. None of the compounds that we tested (methionine, homoserine, homoserine lactone, homoserine thiolactone, homocysteine, *S*-adenosyl-homocysteine, *S*-adenosyl-ethionine, cystathionine, *o*-homoserine phosphate, and homocysteine thiolactone) served as substrates. In reactions containing H_6-TraI, OOACP, and AdoMet, none of the tested compounds detectably inhibited the reaction (*15*). However, homoserine lactone and homocysteine thiolactone reacted inefficiently with OOACP or OOCoA in a TraI-independent manner.

In addition to acylating thiol groups, OOTC should be able to acylate other nucleophiles including primary amines, although at lower rates. We therefore tested the ability of OOTC to chemically acylate other compounds. Incubation of OOTC with homoserine lactone produced AAI. Incubation of OOTC with homocysteine thiolactone also produced a similar compound (possibly 3-oxo-octanoyl-homocysteine thiolactone), which also activated TraR. Similarly, incubation of OOTC with AdoMet produced AAI. This nonenzymatic reaction may provide insights about TraI-mediated AAI synthesis. Chemical or enzymatic synthesis of AAI from AdoMet requires two steps: acylation of the amino group of AdoMet and lactonization. Acylation of the amino group and the consequent loss of its positive charge enhances the nucleophilicity of the carboxyl group. We propose that this enhanced nucleophilicity may facilitate ring closure (Fig. 4). The enzyme mechanism is likely to be a ping-pong reaction, in which OOACP serves as the first substrate, which produces the enzyme intermediate 3-oxo-octanoyl-TraI (OOTraI). This hypothesis is supported by the result that chemically acylated TraI (OOTraI) can react with AdoMet to form AAI in the absence of OOACP (*24*).

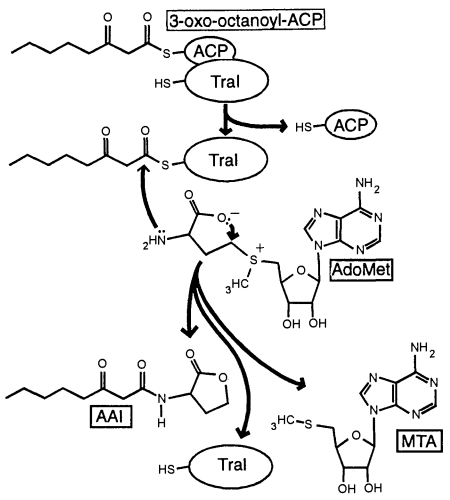

Fig. 4. A model to describe AAI biosynthesis. TraI acquires a 3-oxo-octanoyl group from 3-oxo-octanoyl ACP and catalyzes formation of the amide bond between the amino group of AdoMet and C-1 of the fatty acid. This reaction is followed by lactonization, creating AAI and 5′-methylthioadenosine (MTA). The covalent acyl-TraI intermediate was first proposed by Baldwin and co-workers (*2*), and the formation of this intermediate is supported by experiments in which OOTC was used to acylate TraI (*24*).

REFERENCES AND NOTES

1. R. Wirth, A. Muscholl, G. Wanner, *Trends Microbiol.* **4**, 96 (1996); B. L. Bassler and M. R. Silverman, *Two Component Signal Transduction*, J. A. Hoch and T. J. Silhavy, Eds. (American Society for Microbiology, Washington, DC, 1995), chap. 28; G. P. C. Salmond, B. W. Bycroft, G. S. A. B. Stewart, P. Williams, *Mol. Microbiol.* **16**, 615 (1995); W. C. Fuqua, S. C. Winans, E. P. Greenberg, *J. Bacteriol.* **176,** 269 (1994).
2. D. M. Sitnikov, J. B. Schineller, T. O. Baldwin, *Mol. Microbiol.* **17**, 801 (1995).
3. A. Eberhard *et al.*, *Biochemistry* **20**, 2444 (1981).
4. Y. Y. Adar and S. Ulitzur, *J. Biolumin. Chemilumin.* **8**, 261 (1993); B. L. Hanzelka and E. P. Greenberg, *J. Bacteriol.* **177**, 815 (1995).
5. H. B. Kaplan and E. P. Greenberg, *J. Bacteriol.* **163**, 1210 (1985).
6. W. C. Fuqua and S. C. Winans, *ibid.* **176**, 2796 (1994).
7. ———, *Mol. Microbiol.*, in press; L. Zhang, P. J. Murphy, A. Kerr, M. E. Tate, *Nature* **362**, 446 (1993); K. R. Piper, S. B. Beck von Bodman, S. K. Farrand, *ibid.*, p. 448; I. Hwang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4639 (1994).
8. J. P. Pearson *et al.*, *Proc. Natl. Acad. U.S.A.* **91**, 197 (1994).
9. A. Eberhard, T. Longin, C. A. Widrig, S. J. Stranick, *Arch. Microbiol.* **155**, 294 (1991).
10. G. W. Huisman and R. Kolter, *Science* **265**, 537 (1994).
11. J. G. Cao and E. A. Meighen, *J. Bacteriol.* **175**, 3856 (1993).
12. We constructed pJS101 by the polymerase chain reaction (PCR) amplification of *traI*, using the primers 5′-GCCGCTAGCATGCTGATTCTGACCGTCTCGCC-3′ and 5′-GCCGGATCCTCACGCCGCACTCCTCAACGGGC-3′. The product was digested with Nhe I and Bam HI, and introduced into plasmid pRSETA (Invitrogen). Strain BL21/DE3(pJS101) was cultured to mid-log phase with aeration in 300 ml of LB medium at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and growth was continued until the early stationary phase. Cells were harvested by centrifugation, resuspended in 4 ml of lysis buffer [50 mM NaH_2PO_4, 500 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, 1 mM NaN_3 (pH 7.8)], and disrupted with a French press minicell (20,000 pounds per square inch). The lysate was centrifuged at 33,000*g* for 20 min, and the pellet fraction was washed once in lysis buffer and resuspended in 0.1 M phosphate buffer (pH 7.5) containing 6 M guanidine hydrochloride. Insoluble particles were removed by centrifugation at 150,000*g* for 30 min. The supernatant was loaded at 0.5 ml min^{-1} onto a column (1 cm by 10 cm) containing His*Bind nickel chelation resin (Novagen), which had been equilibrated with the same buffer. We washed bound proteins using 10 column volumes of the same buffer (0.5 ml min^{-1}), after which this buffer was replaced over a 15-min period (1 ml min^{-1}) with a buffer containing 6 M urea and 500 mM NaCl, 20% glycerol, 15 mM tris-HCl (pH 7.4). This buffer was then replaced over a 2-hour period (1 ml min^{-1}) with a native buffer containing 500 mM NaCl, 20% glycerol, 15 mM tris-HCl (pH 7.4). We eluted H_6-TraI using the same native buffer containing 500 mM imidazole. The resulting preparation was judged to be about 90% pure, containing only one detectable contaminant. Dithiothreitol (DTT) and EDTA (1 mM each) were added before freezing at −80°C.
13. The cell-free supernatant of *E. coli* BL21/DE3(pJS101) was assayed for AAI as described in Table 1.
14. We size-fractionated purified H_6-TraI using a Superdex-75 gel filtration column (Pharmacia) at 0.5 ml min^{-1}. Fractions were assayed for AAI synthesis activity as described in Table 1.
15. M. I. Moré *et al.*, unpublished data.
16. K. Magnuson, S. Jackowski, C. O. Rock, J. E. Cronan Jr., *Microbiol. Rev.* **57**, 522 (1993).
17. G. D'Agnolo, I. S. Rosenfield, J. Awaya, S. Omura, P. R. Vagelos, *Biochim. Biophys. Acta* **326**, 155 (1973); G. D'Agnolo, I. S. Rosenfield, P. R. Vagelos, *J. Biol. Chem.* **250**, 5283 (1975).
18. M. Siggaard-Andersen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11027 (1994).
19. T. Ouyang and D. R. Walt, *J. Org. Chem.* **56**, 3752 (1991).
20. J. E. Cronan Jr. and A. L. Klages, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5440 (1981).
21. C. O. Rock and J. E. Cronan Jr., *Methods Enzymol.* **71**, 341 (1981).
22. To determine the K_M for AdoMet, we incubated 3.4 ng of H_6-TraI with 1.4 μM OOACP and a range of AdoMet concentrations from 0.5 μM to 500 μM in a buffer containing 100 mM NaCl, 65 mM tris-HCl (pH 7.4), 5% glycerol, 0.1 mM DTT, and 2 mM EDTA (40 μl total). To determine the K_M for OOACP, we incubated 3.4 ng of H_6-TraI with 500 μM AdoMet and a range of OOACP concentrations from 2.75 nM to 2.75 μM. We assayed AAI as described in Table 1 and analyzed it using double-reciprocal (Lineweaver-Burk) plots.
23. We incubated 68 mM CoA and 120 mM OOTC (final concentrations) in a buffer containing 0.1 M phosphate (pH 7.4) for 3 hours. The reaction was terminated by acidification, and we chromatographed the products by HPLC using a C-8 reversed-phase column (Phenomenex) and a gradient of methanol and 0.5 M phosphate buffer (pH 5.5) from 20:80 to 90:10 (16 min, 2 ml min^{-1}). The peak fraction was dried by rotoevaporation and characterized by proton NMR (D_2O). The spectrum obtained was similar to that of a CoA control but contained additional resonances characteristic of the acyl chain.
24. M. I. Moré *et al.*, in preparation.
25. J. Tempé, A. Petit, M. Holsters, M. Van Montagu, J. Schell, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2848 (1977).
26. C. Fuqua and S. C. Winans, *J. Bacteriol.* **178**, 435 (1996).

27. We thank P. Greenberg and T. Baldwin for helpful discussions and J. Helmann, V. Stewart, and J. Shapleigh for critical reading of this manuscript. We thank J. B. Schineller for providing *o*-homoserine phosphate. Supported by NIH grant GM42893 and by Dana Summer fellowships to L.D.F.

24 January 1996; accepted 22 April 1996