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A Kuspa, L Plamann and D Kaiser
J. Bacteriol. **1992, 174(10):3319.**

JOURNAL OF BACTERIOLOGY, May 1992, p. 3319–3326
0021-9193/92/103319-08$02.00/0

Vol. 174, No. 10

Identification of Heat-Stable A-Factor from *Myxococcus xanthus*

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Received 14 November 1991/Accepted 5 March 1992

The *asg* mutants of *Myxococcus xanthus* fail to produce a set of related substances called A-factor. A-factor is released into the medium and is required early in fruiting body development. Lacking A-factor, the *asg* mutants are defective in aggregation, sporulation, and expression of most genes whose products appear later than 1 h after development is induced by starvation. Previous work has shown that these defects are reversed when A-factor, released by developing wild-type cells, is added to *asg* mutant cells. Part of the material in conditioned medium with A-factor activity is heat stable and dialyzable. This low-molecular-weight A-factor consists of a mixture of amino acids and peptides. Fifteen single amino acids have A-factor activity, and 11 of these are found in conditioned medium. Mixtures of amino acids have a total activity approximately equal to the sum of the activities of their constituents. Conditioned medium also contains peptides with A-factor activity. Pure peptides have A-factor activity, and their specific activities are equal to or less than the sum of the activities of their constituent amino acids. There is no evidence for a specialized A-factor peptide in conditioned medium, one with a specific activity greater than the sum of its constituent amino acids. About half of the heat-stable A-factor activity in conditioned medium can be accounted for by free amino acids, and the remaining half can be accounted for by peptides. It is argued that heat-stable A-factor induces A-dependent gene expression not by the nutritional action of amino acids but through a chemosensory circuit.

Members of the *asg* class of developmental mutants of *Myxococcus xanthus* are deficient in aggregation and sporulation and thus fail to form fruiting bodies (9). In addition, they fail to express β-galactosidase from a series of *lacZ* transcriptional fusions (the *asg*-dependent fusions) that normally are active after 1 to 2 h of development (10).

Developing wild-type cells release substances that can restore the capacity of *asg* mutants to develop; *asg* mutant cells release such substances in 10-fold-lower amounts. Aggregation, sporulation, and β-galactosidase expression are all restored by conditioned medium from wild-type cells undergoing development in suspension (10). This rescue is not due to an auxotrophy of *asg* mutants with cross-feeding by wild-type cells, because *asg* mutants grow in unsupplemented A1 minimal medium (1, 16) at rates similar to those of wild-type cells (9). The substances in conditioned medium that rescue *asg*-dependent gene expression are known collectively as A-factor. A-factor can be assayed by measuring the β-galactosidase synthesis restored to an *asg* mutant that contains the earliest *asg*-dependent *lacZ* transcriptional fusion, Ω4521. About half of the A-factor in conditioned medium has a high molecular weight and is heat labile (15); the remaining half is heat stable and, as indicated by the fact that it passes through dialysis tubing, has a low molecular weight. In this paper we show that the low-molecular-weight material is a mixture of amino acids and peptides.

MATERIALS AND METHODS

Myxobacterial strains. *M. xanthus* DK4322, DK4323, and DK4324 are the *asg*$^+$, *asgA476* mutant, and *asgB480* mutant strains, respectively, which contain the developmentally regulated *lacZ* fusion Tn*5 lac* Ω4521 in a DK101 (*sglA1*) background (10) and are used in the standard A-factor assay (9). DK1622 (5) and DK4399 (10) were used as wild-type strains for the production of crude A-factor. DK4398 is an *asgB480* mutant backcrossed into the DK1622 (*sglA*$^+$) background (10).

Production and determination of A-factor. The A-factor assay and A-factor production were as described previously (9, 10, 15). For the present study, crude A-factor (conditioned medium) was prepared by shaking wild-type cells at a density of 5×10^9 cells per ml for 2 to 4 h in MC7 medium (morpholinepropanesulfonic acid [pH 7.0], $CaCl_2$) and then removing the cells by centrifugation. Crude A-factor was heated at 100°C for 10 min, and then the heat-stable A-factor activity or amino acid content was determined. Dose-response curves for amino acids, peptides, and heat-stable A-factor were constructed by using the appropriate dilutions of each substance in the standard A-factor assay. One unit of A-factor activity is defined as the amount required to stimulate the test cells to produce 1 U of β-galactosidase activity (1 nmol of *o*-nitrophenol per min) above the background activity.

Partially purified heat-labile A-factor was the flow-through fraction of a DEAE cellulose (Whatman) column loaded with crude A-factor that had been dialyzed in MC7 (8).

Paper chromatography. Paper chromatography (23) was used to estimate the amino acid content of heated crude A-factor fractions and to purify biologically active materials. Samples were concentrated 10-fold by lyophilization, suspended in distilled water, applied in spots of 50 μl (added in aliquots of 5 μl each) to Whatman 3MM chromatography paper, and developed in a descending paper chromatography chamber with butanol-acetic acid-water (100:22:50, vol/vol/vol). Paper chromatograms were dried for at least 5 h in a fume hood and then either stained with ninhydrin or cut into strips and soaked with MC7 buffer for 1 h at room temperature to elute small molecules (4.5 cm of paper per 2 ml of buffer). The A-factor activity from each of the strips was determined by the A-factor assay described above. Control experiments showed that the A-factor activity of pure amino

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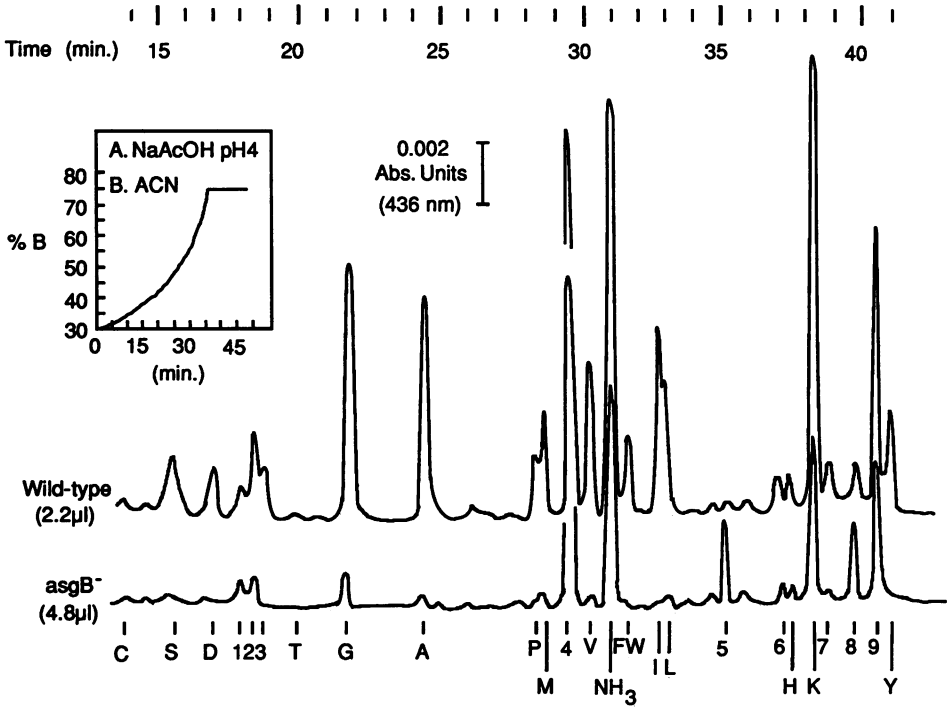

FIG. 1. HPLC detection of amino acids in conditioned medium. Crude A-factor was prepared from wild-type (DK4399) or *asgB* mutant (DK4398) cells from cell supernatants harvested after 2 h of development in shaken suspension (see Materials and Methods). The fractions were heated (100°C, 10 min) immediately after harvesting to inactivate proteases, and free amino acids were derivatized with DABS-Cl and separated by reversed-phase chromatography. Two tracings are shown in which the horizontal axis is time in minutes and the vertical axis is A_{436}. The upper tracing was obtained from 2.2 µl of a wild-type crude supernatant, and the lower tracing was obtained from 4.8 µl of the *asgB* mutant supernatant. The inset displays a profile of the eluting solvent with 43.5 mM sodium acetate (pH 4) as solvent A and acetonitrile as solvent B. Amino acid peaks are identified at the bottom in the single letter code for amino acids. Major unidentified peaks are labeled 1 through 9.

acids could be eluted quantitatively, whereas only about 30% of the activity of the peptide leucine enkephalin could be eluted. This peptide migrated with a relative mobility greater than that of any amino acid. Leucine enkephalin and other peptides were purchased from Sigma Chemical Co.

Dabsylation of amino acids. Amino acids were allowed to react with 4-dimethyl-aminoazobenzene-4′-sulfonyl chloride (DABS-Cl; Sigma) as described previously (3) to produce yellow-orange products. Recrystallization of the DABS-Cl before use (3) was found to be essential. This method can provide very accurate quantitation, because the concentration of DABS-Cl used to make the standards is measured spectrophotometrically and it reacts quantitatively with amino acids in solution. Thus, most of the amino acid standards are derivatized under reaction conditions in which the amount of DABS-Cl is limiting, and >99% of the DABS-Cl reacts with the amino acids (no DABS-Cl or DABS-ONa, the hydrolysis product, can be detected by high-performance liquid chromatography [HPLC] after the reaction). Three amino acids, lysine, tyrosine, and histidine, that react with two equivalents of DABS-Cl were exposed to an excess of DABS-Cl to ensure production of the bisDABS-amino acid derivative. Samples for which the amino acid levels were to be determined were exposed to a concentration of DABS-Cl that was empirically determined to be at least 10-fold greater than the free amino acid level in the sample. This concentration was determined by reacting the samples with increasing amounts of DABS-Cl until 10 times more DABS-ONa was produced than dabsylated amino acids, as measured by HPLC (see below). Beyond that concentration, additional DABS-Cl produced no measurable additional quantity of dabsylated amino acids. To test whether non-amino acid components of the crude fractions interfere with subsequent chromatography steps, dabsylated amino acids were purified from several samples of unknown composition by extraction into diethyl ether (25) and analyzed by HPLC along with nonextracted samples. Since little

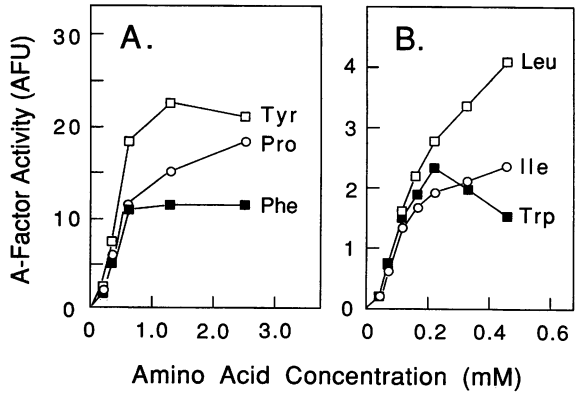

FIG. 2. A-factor activities of single amino acids. Note differences in scales in panels A and B. A-factor units (AFU) are defined in Materials and Methods.

TABLE 1. A-factor activities of amino acids and their concentrations in crude A-factor preparations

Amino acid[a]	A-factor activity (U/ml) at 100 μM[b]	Concn in crude fraction (μM)[c]	Calculated A-factor U/ml in crude fraction[d]
Tyr	5.5	15	0.82
Pro	3.8	13	0.5
Phe	3.8	22[e]	0.75[f]
Trp	3		
Leu	3	55[e]	1.58[f]
Ile	2.8		
Gln	1.2	<0.20	
Asn	1.2	<0.20	
Ala	1.2	55	0.7
Ser	0.8	44	0.32
His	0.5	3.8	0.02
Gly	0.2	83	0.21
Asp	0.18	33	0.06
Glu	0.18	<0.2	0
Arg	0.12	<0.2	0
Val	<0.1	33	0
Met	<0.1	27	0
Thr	<0.1	1.1	0
Lys	<0.1	110	0
Cys	<0.1	5.5	0
Totals	27.5	500	5

[a] The amino acids are listed in order of decreasing A-factor activity.

[b] A-factor activity was measured in the standard A-factor assay. The activity produced for each amino acid at 100 μM was interpolated from the linear portion of a dose-response curve.

[c] The concentration of each amino acid in crude A-factor separated by reverse-phase HPLC is shown. Five independent determinations with five different preparations were averaged; they varied from each other by less than 20%. Crude A-factor is medium conditioned by wild-type cells shaking and starving in MC7 buffer for 2 h at 32°C.

[d] Values are the A-factor activity expected from each amino acid in crude A-factor. Values were obtained by dividing the product of columns 2 and 3 by 100.

[e] The amino acid pairs phenylalanine-tryptophan and leucine-isoleucine were not well resolved by reversed-phase HPLC in every experiment, and the values for these pairs of amino acids are combined. When these pairs were resolved their constituents were roughly equimolar.

[f] Since the specific A-factor activities for the two members of these pairs (Phe and Trp, Leu and Ile) were similar, their average was multiplied by their total concentrations.

Zdifference was found between the extracted and nonextracted samples in the measured concentrations of amino acids, amino acids were dabsylated in crude fractions and were not routinely purified before analysis.

Quantitation of free amino acids. Free amino acids were quantitated from dabsylated samples analyzed on a Waters Associates HPLC system equipped with an analytical (5-μm bead size), C-18 silica reversed-phase column (0.45 by 25 cm, Vydac Type 201HS; Sep/a/ra/tions Group, Mohave, Calif.). The HPLC system consisted of two 6000A pumps, a 720 system controller, a U6K universal injector, a model 440 detector equipped with a filter (436-nm fixed wavelength), and a central processor with peak integration capabilities. Dabsylated amino acid standards were grouped into four sets of five amino acids each. The system was calibrated with a set of standards before each unknown was run. Samples were run twice, and nearly identical results were obtained. The column running conditions were as described by Lin (11). Briefly, solvent A was 43.5 mM sodium acetate (pH 4.0) and solvent B was acetonitrile. The column was loaded in 30% solvent B–70% solvent A and eluted with a concave gradient that reached 75% solvent B after 35 min and then stayed at 75% 10 more min. The column was loaded and run at 1.2 ml/min. The detector was usually set at 0.02 absor bance units, full scale, allowing reliable quantitation of less than 10 pmol of individual amino acids (per peak) with a detection limit of less than 2 pmol. Since the dabsyl moiety on each derivatized amino acid accounts for most of the A_{436}, a given area under any peak represents the same concentration for all monodabsylated amino acids (and half that concentration for bis-dabsyl amino acids such as lysine, histidine, and tyrosine). Samples were run so that at least 10 pmol could be measured in each peak.

RESULTS

Isolation of heat-stable A-factor. A-factor is released from cells shaken in suspension, starting 1 to 2 h after starvation and proceeding over the next several hours (8, 10). Sedimentation of cells from such a suspension gives a clear supernatant (the crude A-factor fraction) that includes both heat-stable and heat-labile forms of A-factor (8, 15). Heat-labile A-factor has been shown to include at least two proteases (15). To identify molecules with heat-stable A-factor activity, knowing that they are dialyzable and probably of low molecular weight, the supernatant fluid from a shaken cell suspension was analyzed by paper chromatography (23). Half of a vertical chromatogram with pairs of alternating sample lanes and lanes of amino acid standards was stained with ninhydrin. Most of the ninhydrin-positive material in crude A-factor migrated with the amino acid standards. An equivalent but unstained half of the same chromatogram was cut horizontally into segments; each segment was eluted with MC7 buffer, and each eluate was assayed for A-factor activity. About half of the A-factor activity recovered from

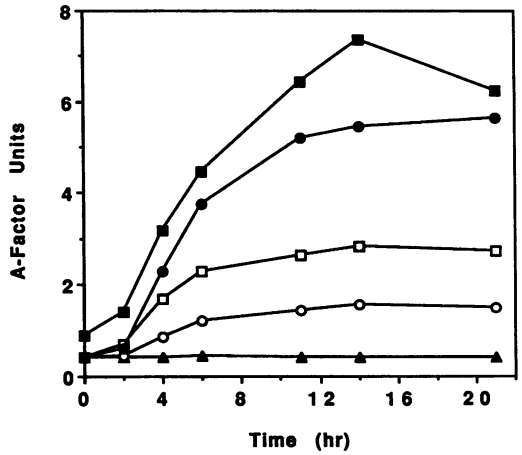

FIG. 3. Developmental time course of β-galactosidase expression from the transcriptional *lacZ* fusion Ω4521. The standard A-factor assay conditions were employed, and the microtiter plates were incubated at 32°C for various times. At each time point a plate was frozen at −20°C. When all samples had been frozen, the plates were thawed and β-galactosidase activities were measured. Background A-factor activity in the test cells (DK4324 in MC7 alone) was not subtracted from the activities of the test cells with added proline, cysteine, or heat-labile A-factor. The developmental time courses of β-galactosidase expression from Ω4521 in the *asg* mutant DK4324 (○), DK4324 plus 200 μM cysteine (▲), DK4324 plus 1 to 2 U of purified heat-labile A-factor (15) (□), DK4324 plus 200 μM proline (●), and the *asg*$^+$ strain DK4322 (■) are shown.

the paper migrated to the positions corresponding to particular amino acids; the eluates corresponding to the tyrosine, proline, and phenylalanine segments showed the highest activities.

Because A-factor biological activity is found in medium conditioned by starving *asg*$^+$ cells, but at a level 10-fold lower in medium conditioned by *asg* mutant cells (10), a quantitative comparison of amino acids released by wild-type cells and mutant cells was made. A crude A-factor preparation was reacted with DABS-Cl, and the dabsylated products were separated by reversed-phase HPLC. Chromatographic effluents of medium conditioned by *asg*$^+$ and *asg* mutant cells are shown for comparison in Fig. 1. Sixteen amino acids present in the *asg*$^+$ preparation were found at least 10-fold lower in concentration in the *asgB480* mutant (Fig. 1). Conditioned medium from *asg* mutant cells contained Tyr, Pro, Phe, Trp, Leu, and Ile at concentrations ranging from 0.3 to 1.0 μM (averages of three preparations). Conditioned medium from *asg*$^+$ cells contained these amino acids at concentrations of 11 to 22 μM (averages of five crude A-factor preparations).

Several dabsyl-labeled peaks that did not correspond to any standard amino acid are designated 1 through 9 in Fig. 1. They represent small molecules that react with DABS-Cl and have chemical properties similar to those of the amino acids. They may represent rare amino acids or other amino compounds. Most of these unidentified substances were present at similar levels in the *asgB* mutant and the *asg*$^+$ strain and thus do not appear to be relevant to A-factor. The levels of substances 3 and 7 are lower in the mutant, but their identification is left for the future. In any case, peaks 1 through 9 separated from the 16 identified amino acids and did not interfere with their quantitation.

Overall, the HPLC showed more than 10-fold-higher amino-acid release from *asg*$^+$ cells than from *asg* cells. This parallels the concentrations of A-factor released into conditioned medium by *asg*$^+$ cells, which were more than 10-fold higher than those in medium conditioned by *asg* mutant cells. To follow up on this parallel, each of the 20 common amino acids was tested for A-factor activity by measuring β-galactosidase produced in DK4324 test cells. (These cells are *asgB480* [Ω4521].) Figure 2 shows dose-response curves for six amino acids. Specific activities for all the common amino acids (taken from the linear parts of the dose-response curves for each of the amino acids, such as those shown in Fig. 2) are summarized in Table 1. The abundance of each amino acid in conditioned medium (crude fraction), as determined by HPLC, is also listed in Table 1. It is evident in Fig. 2 that, when presented at concentrations above their abundance in conditioned medium, Tyr, Pro, Phe, and Leu induce β-galactosidase activity levels that are even higher than that induced by the medium conditioned by *asg*$^+$ cells under standard conditions (about 2 A-factor U).

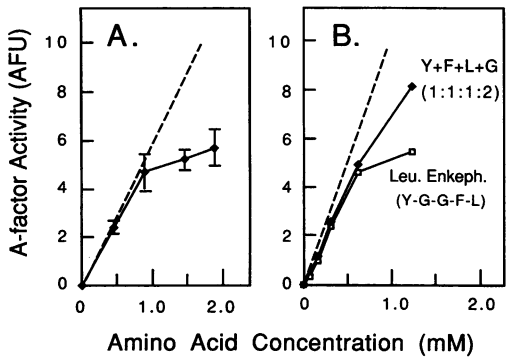

FIG. 4. A-factor activities of combinations of amino acids and peptides. (A) An equimolar mixture of the 19 common amino acids (all but cysteine) was assayed for A-factor activity. The sum of the concentrations of all of the added amino acids is shown. The mean activities and standard errors are given for four independent experiments. The dotted line shows the total activity from a sum of specific activities given in Table 1 (11 A-factor units would be expected from an equimolar mixture of 19 amino acids at a total concentration of 1.9 mM). (B) The A-factor activity of the pentapeptide leucine enkephalin (Y-G-G-F-L) is shown (□). Its concentration is given as the sum of its constituent amino acids (i.e., 1 mM amino acid concentration refers to 0.2 mM peptide). The A-factor activity of the peptide is compared to the activity elicited by an amino acid mixture (◆) that mimics the composition of the peptide (i.e., a 1:1:1:2 mixture of Tyr, Phe, Leu, and Gly). The dotted line shows the activity curve expected for the same amino acid mixture calculated from the specific activities in Table 1.

TABLE 2. Substances without A-factor activity[a]

Substance	Range of concns tested	
	Low	High
Adenosine	60 nM	1 mM
D-Alanine	2.5 mM	8 mM
Diaminopimelic acid	2.5 mM	8 mM
Glucosamine	1 mM	10 mM
Galactosamine	1 mM	10 mM
Glucose	1 mM	10 mM
Galactose	1 mM	10 mM
Ribose	1 mM	10 mM
Rhamnose	1 mM	10 mM
Mannose	1 mM	10 mM
N-Acetylglucosamine	37 μM	3 mM
N-Acetylgalactosamine	37 μM	3 mM
2-Keto-3-deoxyoctonic acid	30 μM	3 mM
Pyruvic acid	1 mM	10 mM
NH_4OH	60 nM	10 mM
$MgSO_4$	0.1 mM	8 mM
$MgCl_2$	10 μM	20 mM
$CaCl_2$	10 μM	20 mM

[a] All substances were tested in a standard A-factor assay. No A-factor activity was detected for any substance listed at the range of concentrations tested.

No A-factor activity was detected from low-molecular-weight monomeric substances other than amino acids. Table 2 lists the metabolites of *M. xanthus* and salts that were tested. Pyruvate, ammonium ion, and sulfate ion were inactive. They are effective carbon, nitrogen, and sulfur sources, respectively, for *M. xanthus* (1). Also devoid of A-factor activity were adenosine, which is released by developing cells and which can lower the minimum cell density required for aggregation (21), glucosamine and galactosamine, which can rescue fruiting body formation by *csgA sglA* double mutants (4), and constituents of peptidoglycan, which can induce rippling (22). Several sugars and amino sugars that are components of *Myxococcus* polysaccharides (18, 24) showed no A-factor activity. Furthermore, none of the organic compounds listed in Table 2 inhibited the activity of crude A-factor when mixtures were assayed (data not shown).

To examine the relevance of the A-factor activity of amino acids to normal development, the time course of β-galactosidase production by an *asg* strain exposed to an amino acid with high A-factor specific activity was compared with that

of an *asg*$^+$ strain without amino acid addition (Fig. 3). In the *asg*$^+$ strain with no amino acid added, an increase in β-galactosidase activity was observed beginning at about 2 h. The activity rose and then became level around 10 h, in keeping with earlier observations (7). The β-galactosidase activity of the *asgB480* strain (with no amino acid added) increased only a few units, as expected because it releases about 5% the A-factor activity of *asg*$^+$ (10). The addition of proline to the *asgB480* strain increased the level of expression almost to that of the *asg*$^+$ strain (without proline). This proline-rescued activity followed the same time course as that in *asg*$^+$ development. Thus the ability of an *asg* mutant to respond with β-galactosidase production to the addition of proline resembles in time course the response of an *asg*$^+$ strain to endogenous A-factor. The addition of purified heat-labile A-factor (15) to the *asg* mutant also produced a similar time course of β-galactosidase production (Fig. 3).

Strain DK4324 (*asgB480*) was used in the standard A-factor assay. However, DK4323, which contains an *asg* mutation (*asgA476*) at a different locus (9), gave the same response to amino acids measured by β-galactosidase production (data not shown). Thus, more than one kind of *asg* mutant can respond to the addition of amino acids.

Activity of amino acid mixtures and peptides. The A-factor activities of mixtures of amino acids corresponded to the sums of the activities of the amino acid constituents. No evidence for synergy between amino acids was found, nor was there evidence for inhibition by any amino acid, with the exception of cysteine (see below). Figure 4A shows the A-factor activity of an equimolar mixture of 19 amino acids (those listed in Table 1, excluding cysteine). The total activity for the 19 amino acids was found to be close to the sum of the specific activities of individual amino acids listed in Table 1. The response to increasing concentration of individual amino acids (Fig. 2) tended to depart from linearity in the 0.15 to 0.7 mM concentration range. The exact saturating concentration depended on the amino acid. Since the specific activity of each amino acid was calculated from the linear part of its dose-response curve, correspondence of total activity with the sum of specific activities might be expected only for levels below the saturating concentration range. As expected, a break in the dose-response curve for the amino acid mixture was observed around 1 mM total concentration (Fig. 4A).

Cysteine inhibited A-factor activity. It antagonized the A-factor activity of added amino acids: cysteine at 100 μM inhibited by 50% the ability of an equimolar mixture of the other 19 amino acids to rescue β-galactosidase production. With 250 μM cysteine, rescue activity of the mixture of 19 was totally abolished. Cysteine depressed the residual A-factor activity of the *asgB480* mutant (Fig. 3). This inhibitory effect of cysteine appears to be unrelated to its reducing power, because reducing agents or molecules with structures related to cysteine (dithiothreitol, reduced glutathione, cystathionine, homoserine, homocysteine, homocystine, and cystine) had no inhibitory activity (data not shown).

In the paper chromatographic experiment described above, significant A-factor activity migrated ahead of the common amino acids to a region where short hydrophobic peptides would migrate. The A-factor activity was distributed diffusely in this region of the chromatogram, and no ninhydrin staining was detected there, suggesting that a variety of peptides might be active. Eleven defined peptides were assayed; all had A-factor activity (Table 3). The pentapeptide leucine enkephalin at a low concentration was found to elicit the same β-galactosidase response as a mixture of its constituent amino acids (tyrosine, glycine, phenylalanine, and leucine in a 1:2:1:1 ratio; Fig. 4B). Moreover, the observed β-galactosidase expression from this mixture of single amino acids was close to that calculated from the specific activities of the individual amino acids. Di- and tripeptides were examined (Table 3). Some of the peptides tested contain only amino acids with high A-factor specific activity, some contain only amino acids with low A-factor specific activity, and some contain amino acids with both high and low (specific) activities. Each peptide had an A-factor activity close to or less than the combined activity of its constituent amino acids (Table 3), as if each active amino acid in a peptide was making a contribution. Native proteins may not have A-factor activity; neither bovine albumin nor immunoglobulin G was active at 0.8 to 80 μg/ml.

TABLE 3. A-factor activities of peptides

Peptide	A-factor activity (U/ml) of:	
	Peptide[a]	Constituent amino acids[b]
Tyr-Gly-Gly-Phe-Leu	12	12.7
Tyr-Gly-Gly	9	5.9
Pro-Phe	6	7.6
Trp-Gly-Gly	4	3.4
Phe-Pro	3	7.6
Pro-Gly	2.75	4.0
Lys-Leu	1.75	3.0
Met-Ala-Ser	1.5	2.0
Val-Gly-Gly	0.5	0.4
Met-Glu	0.75	0.18
Asp-Asp	0.18	0.36

[a] The specific activity of the peptide present in the A-factor assay at 100 μM is shown. The activity was interpolated from the linear portion of a dose-response curve. The range of peptide concentrations tested was 10 to 600 μM.

[b] These values were calculated by adding the specific activities of each amino acid contained in each peptide. The amino acid activities at 100 μM were taken from Table 1. For example, for Tyr-Gly-Gly, the calculation was 5.5 (Tyr) + 0.2 (Gly) + 0.2 (Gly) = 5.9 A-factor U/ml.

Since *M. xanthus* efficiently uses amino acids as sources of carbon and energy (20), a mixture of amino acids that includes all amino acids essential or limiting for growth (namely, Val, Leu, Ile, and Phe) might allow growth of the cells. Accordingly, the effects of the addition of amino acids on the growth and β-galactosidase expression of starving DK4324 (*asgB480* Ω4521) cells were compared. An increase in total cellular protein was just detectable over the assay period, beginning about 1 mM, the point at which the amino acid mixture dose-response curve breaks from its initial linearity (Fig. 4A). With a 1.9 mM concentration of the added mixture, an increase was first detected in the number of cells by comparison of microscopic cell count at the start and end of the assay. No net increase in protein or cell numbers was detected below these concentrations (data not shown). Casitone, a partial hydrolysate of casein that contains peptides and amino acids, rescued β-galactosidase expression from Ω4521 below concentrations of 0.04% (wt/vol) (Fig. 5). However, when the Casitone concentration exceeded about 0.05% (Fig. 5), the concentration required for an increase in cell number, the rescue of β-galactosidase expression began to fail, falling approximately to the background level when the Casitone concentration reached 0.15 to 0.2%. β-Galactosidase expression from Ω4521 was not observed during vegetative growth (7). Apparently, the addition of amino acid mixtures to DK4324 (*asgB480* Ω4521) induces an increase in β-galactosidase expression up to but

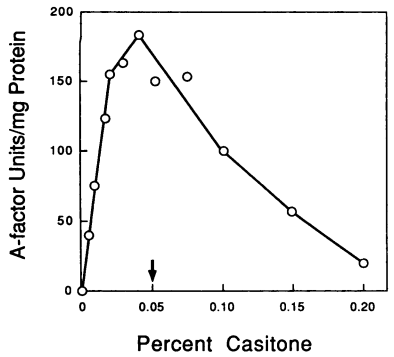

FIG. 5. Effect of Casitone on expression of Ω4521 in the A-factor assay. Casitone, which is a partial hydrolysate of casein and can serve as a complete growth medium for *M. xanthus*, was used as a source of peptides and amino acids in the standard A-factor assay. A-factor units per milligram shows the A-factor activity in each assay well divided by the total amount of protein contributed by the test cells in the well. Since a 10-mg/ml solution of Casitone gave a protein determination of less than 0.04 mg/ml (data not shown), no correction of protein values for Casitone addition was made. The arrow indicates the approximate percentage of Casitone (0.05%) at which an increase in cell number begins.

not beyond the lowest concentration that supports cell growth.

Quantitating the contribution of amino acids to total A-factor activity. Given the A-factor activities of amino acids and their presence in conditioned medium, they should be contributing to the heat-stable A-factor activity of conditioned medium. To assess quantitatively their contribution, heat-stable A-factor release by wild-type cells starving in suspension was compared with amino acid release. Samples of conditioned medium were collected at half-hour intervals after the initiation of starvation in MC7 buffer, each sample was assayed for A-factor by β-galactosidase activity, and amino acids were quantitated by HPLC (Fig. 6). A significant increase in heat-stable A-factor activity was detected by 1 h, and by 3 h it had risen sixfold (Fig. 6), as previously observed (8). The amino acid concentration in conditioned medium began to rise between 1.5 and 2 h; by 3 h it had increased fivefold (Fig. 6). The time course of heat-stable A-factor production paralleled the release of amino acids (Fig. 6).

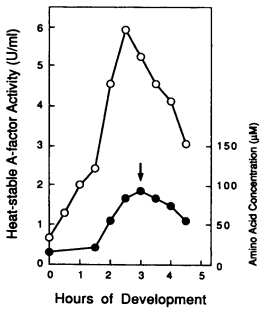

FIG. 6. Time course of release of heat-stable A-factor activity and amino acids. Conditioned medium was heated for 10 min at 100°C before A-factor activity was assayed. Amino acids were quantitated by HPLC as described in Materials and Methods. The scale on the left side of the figure (A-factor units) and the scale on the right side (amino acid concentration) were made commensurate as described in the text by using the 3-h sample for calibration. At 100 μM the six amino acids in the proportions found in the 3-h sample produce about 2 U of A-factor activity per ml; the other mixtures produced A-factor activity in proportion to the total amino acid concentration.

To estimate the fraction of total heat-stable A-factor contributed by amino acids, a mixture of the six most active amino acids at their concentrations in 3-h conditioned medium was made (Fig. 6). The A-factor activity of this reconstructed mixture was 2 U. Since 6 total U of heat-stable A-factor were found at 3 h, the amino acids Tyr, Pro, Phe, Ile, Leu, and Trp accounted for one-third of it (2 U in a total of 6 U of heat-stable A-factor at 3 h).

A second part of this experiment was designed to average out day-to-day variations in the A-factor bioassay in estimating the contribution of amino acids to total heat-stable A-factor. Four independent preparations of crude conditioned medium were harvested at about the same time of development, their A-factor activities were measured, and their amino acid contents were determined by HPLC. These four preparations were typical because they averaged 11 U of heat-stable A-factor per ml compared with a 11.4-U/ml average for 19 large-scale crude A-factor preparations. Based on the amino acid analyses, four reconstructed mixtures of the same six amino acids (those with the highest specific activities) were prepared in which the molar proportions of the amino acids in each were adjusted to their relative abundance in the four crude A-factor preparations. The A-factor activity of each reconstructed mixture was then measured alongside that of the corresponding heated (100°C, 10 min) crude A-factor preparation. Seven independent comparative bioassays showed that tyrosine, phenylalanine, proline, tryptophan, leucine, and isoleucine accounted for 35 ± 14% of the activity produced by the heated fractions, in quantitative agreement with the experiment of Fig. 6.

DISCUSSION

Two kinds of substances rescue expression of β-galactosidase from the A-factor-dependent *lacZ* transcriptional fusion Ω4521. One is heat labile and has been found to be a mixture of proteases (15); the other is heat stable and has a low molecular weight. The data presented here imply that the heat-stable A-factor is a subset of the amino acids and that peptides are A-factor to the extent that they contain these amino acids. Separation of the constituents of medium conditioned by developing *asg*$^+$ cells, either by paper chromatography or by HPLC, correlated A-factor activity with amino acids and peptides. A-factor activity appears to be confined to amino acids and peptides; no other small molecule tested had A-factor activity. Five amino acids had no detectable ability to induce β-galactosidase from Ω4521 when tested alone. Of the 15 amino acids whose A-factor activity was measurable, 11 were found in conditioned medium. Cysteine antagonized the activity of the other amino acids and repressed the residual β-galactosidase activity in DK4324, an *asgB480* Ω4521 strain.

The specific activities of individual amino acids (Table 1) form an almost continuous spectrum from 5.5 A-factor U for Tyr (at 100 μM) down to 0.12 U for Arg (at 100 μM), an activity that is just above the limit of detection. Abundances of the active amino acids in conditioned medium range from lysine at 110 μM to glutamine at <0.2 μM. Quantitative comparison of the amino acid content and the total heat-stable A-factor activity of conditioned medium showed that the net activities (specific activity times abundance for each

amino acid) of seven amino acids, Pro, Phe, Tyr, Trp, Leu, Ile, and Ala, would account for 38% of the average total measured heat-stable A-factor activity. The sum of the net activities of all the amino acids gives 5 A-factor U per ml (Table 1), or 44% of the average total measured heat-stable A-factor activity.

We suggest that the mixture in conditioned medium of peptides that contain active amino acids accounts for the remaining half (56%) of the heat-stable activity that is not accounted for by individual amino acids. Supporting this suggestion, 95% of the heat-stable A-factor activity in the conditioned medium was found to be dialyzable. A mixture of amino acids and polypeptides with fewer than about 100 amino acid residues would be dialyzable. All 11 peptides tested had A-factor activity. The specific activity of each peptide tested was close to the combined activities of its constituent amino acids. Significant A-factor activity was eluted from a region of the paper chromatogram where peptides would migrate. However, there was no localized spot of biological activity or of ninhydrin staining on this part of the chromatogram, as if the region contained many different peptides. The absence of a localized spot argues against any significant contribution by a specialized A-factor peptide, one with a specific activity greater than the sum of its constituent amino acids.

Rather than a specialized A-factor peptide, the data favor a collection of different peptides, one that could be produced by the action of a mixture of proteases on a mixture of proteins. Total Coomassie blue dye-staining material in conditioned medium is equivalent to more than 120 μg of polypeptide per ml (15). If proteolyzed, this material would give rise to many peptides; any that contain 1 or more of the 15 active amino acids would be expected to have A-factor activity. Plamann et al. (15) associated heat-labile A-factor with a set of proteases. We suggest that these proteases are responsible in vivo for the production of the peptides and amino acids that make up heat-stable A-factor, thus explaining why heat-labile A-factor can either add to or replace heat-stable A-factor.

If amino acids and peptides compose the heat-stable A-factor, how do they result in β-galactosidase expression in an A-factor-dependent *lacZ* fusion strain? Since development is induced by starvation, are the amino acids and peptides serving simply as nutrients for the protein synthesis that is necessary for early *Myxococcus* development? Kuspa et al. (10) showed that the *asgB480* mutation carried by the assay strain does not entail auxotrophy: the mutant strain grows on minimal medium like *asg*$^+$. Furthermore, were the nutritional hypothesis correct, any good nutritional source might be expected to have A-factor activity. On the contrary, pyruvate, which was shown by Bretscher and Kaiser (1) to be an efficient carbon and energy source for *M. xanthus*, has no A-factor activity (Table 2). Moreover, the amino acids lysine, methionine, threonine, and valine, all excellent nutrients that increase the rate of *M. xanthus* growth in minimal medium (1), have no detectable A-factor activity. An alternate but related possibility is that amino acids spare the carbon and energy of starving *asgB480* cells, allowing their residual 5 to 10% of normal A-factor activity (10) to provide the necessary A-signal. Against this possibility, amino acid rescue of β-galactosidase from Ω4521 in the *asg* mutant strain was found to follow the same time course as expression in its *asg*$^+$ counterpart without added amino acids (Fig. 3). The similar time course implies that *asgB480* cells initiate development in the presence of an amino acid like *asg*$^+$ cells that are starving without any added amino acid. High levels of amino acids do permit growth, but growth levels are more than 10-fold higher than those that elicit a measurable A-factor response. Moreover, the higher amino acid levels that permit growth actually depress expression from the *asg*-dependent *lacZ* fusion, Ω4521 (Fig. 5).

Since the A-factor activity of external amino acids and peptides does not simply reflect their nutrient value, this leaves open the possibility that they are a cell-to-cell signal necessary for fruiting body development to proceed beyond its earliest stages (10). Fifteen different amino acids have A-factor activity. How might so many different amino acids be sensed? There could be one chemosensor with various affinities for the different amino acids or several chemosensors, each specific for one or a group of amino acids. There may also be sensors for the peptides that have A-factor activity. Alternatively, peptides might be degraded by periplasmic or outer membrane-bound peptidases to their constituent amino acids, which would be sensed. Degradation seems to be the more likely alternative, since the activity of a peptide measured by β-galactosidase production is close to the sum of the specific activities of its constituent amino acids.

At least two types of cell constituents are known to have the capacity that would be required to differentiate among many amino acids. First, there are amino acid binding and transport proteins. In nature, *M. xanthus* relies on communal extracellular degradation of protein; it feeds on the peptides and amino acids thus produced (17). *M. xanthus* metabolism is based on the utilization of amino acids; sugars are not commonly used as carbon or energy sources (20). It is therefore likely that *M. xanthus* has transport proteins for the efficient uptake of amino acids and peptides. The *spo0K* locus, which encodes oligopeptide permease, is essential for the first stage of sporulation in *Bacillus subtilis* (14, 19). Other amino acid transport proteins could also serve as sensors, as the sugar-binding proteins do for chemotaxis in *Escherichia coli*, *Salmonella typhimurium*, and *B. subtilis* (12, 13). A second type of cell constituent with the required capacity for sensing many amino acids is the machinery for protein synthesis. In *E. coli* and *S. typhimurium*, the lack of a codon-demanded amino-acylated-tRNA is sensed by the ribosome, ppGpp and pppGpp are produced, and a stringent regulatory response is initiated (2).

ACKNOWLEDGMENTS

We thank Craig Zwizinski, who first observed that a fraction of A-factor was heat stable.

This investigation was supported by Public Health Service grant GM23441 from the National Institute of General Medical Sciences. L.P. was an American Cancer Society Postdoctoral Fellow, and A.K. was supported by Public Health Service training grant GM07599 from the National Institutes of Health.

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