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Development and Validation of a Whole-Cell Inhibition Assay for Bacterial Methionine Aminopeptidase by Surface-Enhanced Laser Desorption Ionization–Time of Flight Mass Spectrometry

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Bacterial methionine aminopeptidase (MAP) is a protease that removes methionine from the N termini of newly synthesized bacterial proteins after the peptide deformylase enzyme cleaves the formyl group from the initiator formylmethionine. MAP is an essential bacterial gene product and thus represents a potential target for therapeutic intervention. A fundamental challenge in the antibacterial drug discovery field is demonstrating conclusively that compounds with in vitro enzyme inhibition activity produce the desired antibacterial effect by interfering with the same target in whole bacterial cells. One way to address the activity of inhibitor compounds is by profiling cellular biomarkers in whole bacterial cells using compounds that are known inhibitors of a particular target. However, in the case of MAP, no specific inhibitors were available for such studies. Instead, a genetically attenuated MAP strain was generated in which MAP expression was placed under the control of an inducible arabinose promoter. Thus, MAP inhibition in whole cells could be mimicked by growth in the absence of arabinose. This genetically attenuated strain was used as a benchmark for MAP inhibition by profiling whole-cell lysates for unprocessed proteins using surface-enhanced laser desorption ionization–time of flight mass spectrometry (MS). Eight proteins between 4 and 14 kDa were confirmed as being unprocessed and containing the initiator methionine by adding back purified MAP to the preparations prior to MS analysis. Upon establishing these unprocessed proteins as biomarkers for MAP inhibition, the assay was used to screen small-molecule chemical inhibitors of purified MAP for whole-cell activity. Fifteen compound classes yielded three classes of compound with whole-cell activity for further optimization by chemical expansion. This report presents the development, validation, and implementation of a whole-cell inhibition assay for MAP.

Escherichia coli methionine aminopeptidase (MAP) catalyzes the removal of the initiator methionine from newly synthesized proteins and thus has a direct impact on vital cellular processes (1, 9). Specifically, this enzyme has been shown to be essential for bacterial growth (2), making it a potential target for therapeutic intervention (14). Furthermore, this enzyme appears to be conserved across several bacterial species (9), suggesting that inhibitors of MAP might represent broad-spectrum therapies.

Unlike eukaryotic systems, protein synthesis in bacteria is initiated with a formylmethionine residue (Fig. 1) (9). During elongation of the nascent protein, the formyl group is removed by the action of a peptide deformylase enzyme. Subsequently, the deformylated N-terminal methionine is cleaved to form the mature protein. In the absence of either the deformylase or MAP enzyme, newly synthesized, unprocessed proteins are rendered incapable of folding into functional secondary or tertiary structures. This leads to disruption of cellular processes and results in the arrest of culture growth (2, 10).

A continual challenge in antibacterial research is to advance compounds that specifically inhibit targets in whole bacterial cells. In the early stages of antibacterial drug discovery, an essential gene product (i.e., enzyme) from a pathogen is typically isolated and compound classes that disrupt the enzyme activity are subsequently identified. Once classes of compounds with good inhibitory activity (usually μM to nM) are found, the compounds are incubated with bacterial cultures to determine their relative ability to kill the bacteria or arrest bacterial growth. Unfortunately, neither in vitro inhibition of purified enzyme nor the growth arrest values provide direct evidence to confirm that the bacteria are killed by virtue of the compound penetrating the cell and inhibiting the specific target enzyme. In many cases, a quality in vitro inhibitor for a specific enzyme may inhibit cell growth by some generalized mechanism (i.e., membrane disruption) that may lead to toxicity issues. When this occurs, a compound may get advanced for further consideration during the drug discovery process but later be found to have unacceptable properties during toxicity studies. Thus, a robust screening assay to demonstrate that the compound inhibits the target enzyme in intact bacterial cells would provide a mechanism to discard some compounds with undesirable properties prior to lead expansion and toxicity

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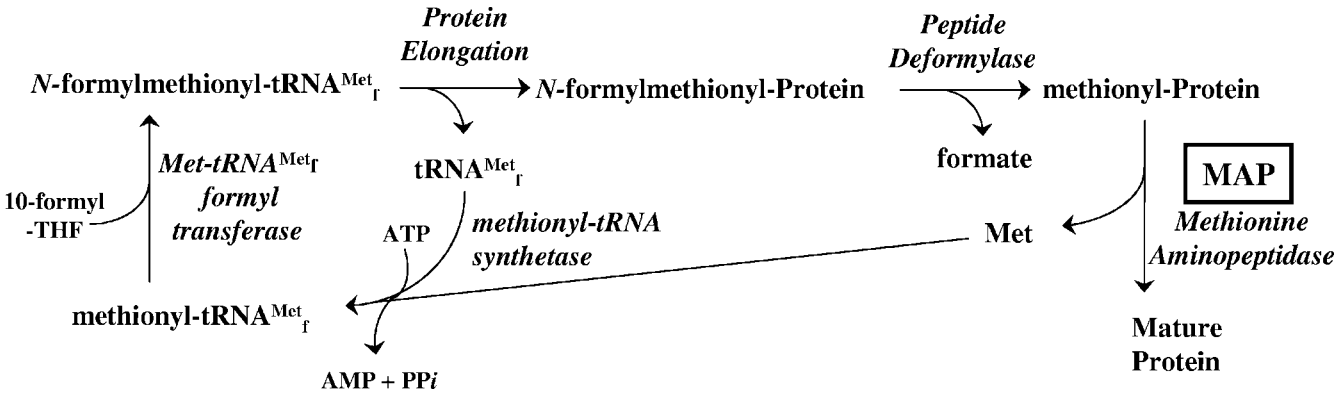

FIG. 1. Role of formylmethionine in bacterial protein biosynthesis. The enzymes involved and utilization of formylmethionyl (Met_f) in the initiation and processing of bacterial proteins as reviewed by Meinnel et al. (9) are shown. THF, tetrahydrofolate.

studies. In the current study, a whole-cell screening assay has been developed, validated, and used to rank order inhibitors of *E. coli* methionine aminopeptidase.

MATERIALS AND METHODS

Bacterial strains and constructs. The wild-type EMG-2 strain of *E. coli* (ATCC 23716) was used as the parental strain for all experiments. The *map* gene of EMG-2 was placed under the control of the arabinose promoter by recombination using a modified version of the methods described previously (5, 8). The procedures were modified in the following way. A complete arabinose BAD promoter with the regulatory *araC* gene amplified from an intact arabinose operon was introduced into the pKO3 vector (with the pKO3 SapI sites having been previously destroyed by site-directed mutagenesis). A SalI site and an engineered SapI site (to allow fusion at the AUG used by the P_{BAD} promoter) preceded the P_{BAD}/*araC* fragment; an *rrnB* transcription terminator and NotI and BglII sites followed the insertion. This generated a vector designated pKO-pro. A region containing the first 500 bp, starting at the initiation codon, of the *map* gene was amplified from genomic DNA and introduced into pKO-pro vector between the SapI and SalI sites. The region downstream of the P_{BAD}/*araC* fragment contained a 500-bp fragment from the intergenic region between the *map* and *rpsB* (ribosomal protein S2) genes. Recombination and selection were performed as described elsewhere (8). The resulting recombinant strain (referred to hereafter as MAPr) was designed to allow MAP expression to be regulated by growth on arabinose so that whole-cell inhibition of the enzyme could be mimicked by removal of arabinose.

Cloning and purification of full-length MAP protein. For antibody generation, full-length MAP was cloned and expressed in *E. coli* strain TOPO IOF′ (QIAGEN). Briefly, the MAP gene was generated from total *E. coli* DNA by PCR using MAP-specific primers (forward primer, ATGGCTATCTCAATCAAG ACC; reverse primer, TTCGTCGTGCGAGATTATCGC). The PCR product was cloned into the pCRT7/CT-TOPO plasmid vector of the pCRT7TOPOTA expression system from QIAGEN according to the manufacturer's protocol. Colonies were selected on Luria broth (LB) agar plates for ampicillin resistance. The correct insertion of the PCR product into the pCRT7/CT TOPO vector was examined by applying PCR using one MAP-specific primer (forward) and one vector-specific primer (T7 reverse priming site). Several clones containing the vector with the PCR fragment in the correct orientation were confirmed by DNA sequencing. Thereafter, BL21 cells were transformed with the TOPO vector containing the correct insert and protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Cloning of the MAP gene lacking the stop codon results in His-tagged protein (tag sequence derived from vector), which was then purified via Ni^{2+} columns according to the manufacturer's protocol (QIAGEN).

Monoclonal antibody preparation. The purified His-tagged MAP protein was separated on an sodium dodecyl sulfate (SDS)-polyacrylamide gel, blotted onto nitrocellulose. and stained with Ponceau red. The band corresponding to the MAP protein was cut out and used as the antigen to generate mouse monoclonal antibodies in an in-house facility (unpublished data).

Culture conditions for the MAP-attenuated (MAPr) strain. The parental strain and the MAPr strain, with the arabinose-regulatable MAP gene, were grown overnight at 37°C on LB agar plates containing 10 mM arabinose. To examine the effect of MAP down-regulation on cells, LB medium without arabinose was inoculated with the overnight cultivated bacteria to an optical density at 600 nm (OD_{600}) of 0.05. Depletion of MAP in the MAPr strain was achieved by sequential transfer of cultures into fresh medium without arabinose at a dilution of 1:10 after reaching an OD_{600} of 0.4. After three rounds of growth and dilution, the residual arabinose and/or MAP protein was diminished to levels that began to alter the culture growth rate. At various time points, 2-ml samples of the cultures were removed and analyzed directly or harvested by centrifugation and frozen at −80°C for further analyses. MAP expression levels in the MAPr strain in comparison to parental cultures were then monitored by immunoblot analysis (see below). Finally, the recovered cells were assessed for morphological changes and viability using microscopic methods (see below) and for MAP inhibition activity by surface-enhanced laser desorption ionization–time of flight mass spectrometry (SELDI-TOF MS) (see below).

Protein determination. Cell pellets were lysed in 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.3% SDS. After incubation at 37°C for 30 min, protein concentrations were determined using the BCA protein assay (Pierce, Rockford, Illinois) according to the manufacturer's protocol.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. For protein separation, the Laemmli system with 10% SDS-polyacrylamide gels was used (6). Briefly, 10 μg of total protein from the whole-cell lysates was used for separation and blotting. Immunoblotting was based on the procedure of Towbin et al. (13) and carried out as described previously (7). Blots were probed with a monoclonal antibody against MAP (described above) and a secondary anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase.

Cell viability staining and fluorescence microscopy. To evaluate whether the decrease in growth rate after arabinose depletion in the MAPr strain or after chemical inhibition of MAP was due to cell death or inhibition of cell division, cell viability was determined by staining with Live/Dead reagent according to the supplier's instructions (Molecular Probes) followed by examination with a fluorescence microscope (Leitz).

MIC determination. The MICs were determined as described previously (11). Briefly, bacterial cultures (5×10^5 CFU/ml) were incubated overnight in brain heart infusion (BHI) broth in the presence of test compounds in duplicate samples in a twofold broth dilution series.

Screening of compounds in whole-cell assays. Several chemical compounds, from both cell-based and purified enzyme screens that were nonlytic to mammalian erythrocytes and had previously been shown (unpublished results) to inhibit MAP activity in vitro (50% inhibitory concentration [IC_{50}] of up to 30 μM) as well as inhibit cell growth (50% effective concentration of <500 μM and MIC of <128 μg/ml), were incubated with 150-μl bacterial cell cultures in 96-well microtiter plates at 37°C to an OD_{600} of 0.3. Next, various concentrations of inhibitors were added (0, 8, 16, 32, 63, 125, 250, and 500 μM), and culture growth was monitored for four hours. Cultures without compounds served as controls. Bacterial cultures were collected, recovered by centrifugation, and frozen at −80°C after 1, 2, 3, and 4 h of incubation in the presence and absence of inhibitors. All tests were carried out in triplicate. Frozen cells were used to screen for MAP inhibition using SELDI-TOF MS (see below).

SELDI-TOF MS. Frozen bacterial culture pellets were lysed with 10 μl B-PER II reagent (Pierce) per ml of culture adjusted to an OD_{600} of 0.1. Metal-binding

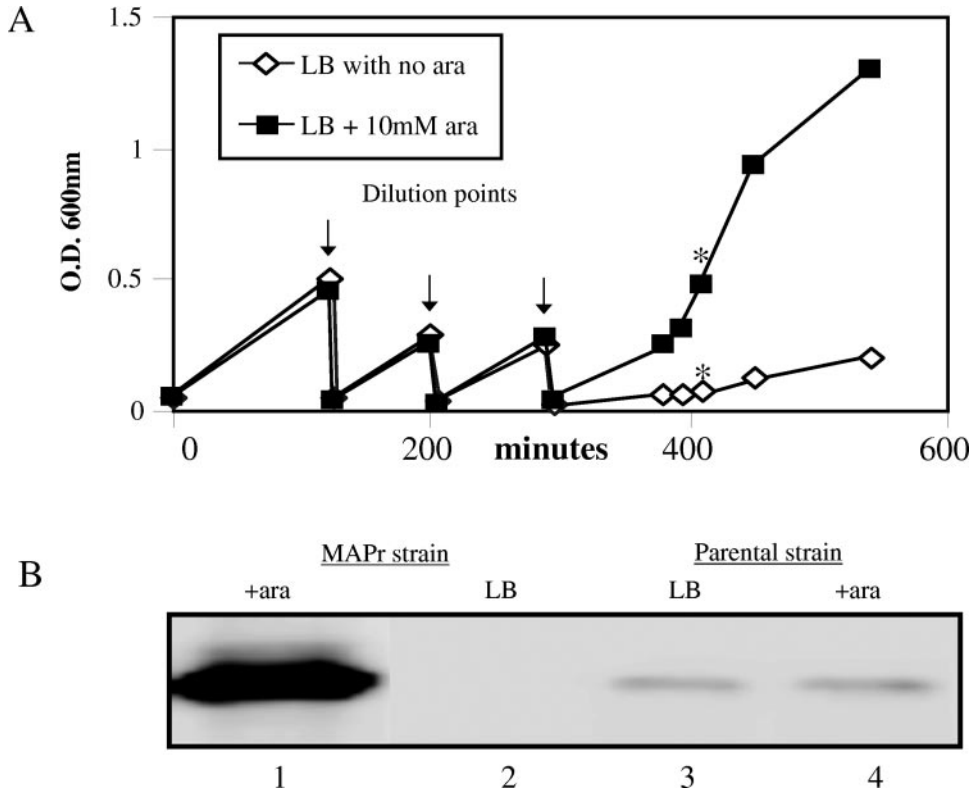

FIG. 2. Growth kinetics and MAP protein expression in the MAPr strain. (A) Cultures of the MAPr strains were sequentially diluted into LB with or without 10 mM arabinose as described in Materials and Methods. After three dilutions and growth in medium without arabinose, the MAPr strain growth kinetics began to diverge (315 min). The time points displayed on the immunoblot in panel B are indicated by asterisks. (B) The relative level of MAP protein expressed from each culture was determined by SDS-polyacrylamide gel electrophoresis and immunoblotting, all performed as described in Materials and Methods. Lane 1, MAPr strain grown in LB plus 10 mM Ara and collected at the 430-min time point indicated in panel A; lane 2, MAPr strain after removal of Ara and collected at the 430-min time point; lane 3, parental strain grown in LB; lane 4, parental strain grown in LB plus 10 mM Ara.

(immobilized metal affinity capture IMAC-Cu) ProteinChips from Ciphergen Biosystems, Inc. (Hercules, CA) were prepared according to the supplier's standard protocol. Briefly, culture lysates were incubated for 30 min at ambient temperature on the IMAC-Cu ProteinChips, washed five times with phosphate-buffered saline and three times with H_2O, air dried, and then loaded twice with 0.5 μl of saturated sinapinic acid matrix dissolved in 50% acetonitrile with 0.5% trifluoroacetic acid. The enriched proteins on the ProteinChip surfaces were detected on a PBS II ProteinChip reader from Ciphergen Biosystems, Inc. using the following instrument parameters: mass range, 3,000 to 15,000 *m/z*; laser intensity, 220 arbitrary units; sub positions, 20; and shots per sub position, 10 to 15. Spectra were normalized by total signal intensity and compared by overlaying profiles using Ciphergen ProteinChip software version 3.0.2.

Enzyme add-back experiments. To confirm that the mass shift of 131 Da detected in proteins from the MAPr strain or in parental cultures incubated with MAP inhibitors was due to unprocessed N-terminal methionine residues, purified, recombinant MAP enzyme was preincubated with the cell lysates prior to spotting the sample onto the SELDI-TOF ProteinChips as described above.

RESULTS

Correlation of induction and repression of MAP with growth kinetics. Bacterial methionine aminopeptidase is the product of the *map* gene and is essential for bacterial growth as judged by the inability to produce viable null mutants (2). A conditional knockout of the *map* gene under the control of the *lac* promoter has been reported in which cells grew only in the presence of a *lac* operon inducer (2). Similarly, Guzman et al. (5) have shown that other essential bacterial genes can be placed under the control of the *araBAD* promoter for growth in arabinose. In the current study, the *map* gene of the EMG-2 strain of *E. coli* was placed under the regulatory control of the *araBAD* promoter such that MAP would be produced only in the presence of arabinose. The initial evaluation of this MAP-regulatable strain (MAPr) was targeted at determining whether the expression of MAP could be turned down selectively to mimic the actions of a potent and selective drug. The effect of variable *map* expression was evaluated by growth kinetics and by immunoblotting. An attempt to grow the MAPr strain in medium containing no arabinose was unsuccessful, demonstrating that synthesis of the *map* gene product was under tight control of the *araBAD* promoter. Next, MAP expression levels were evaluated in parental and MAPr strains in the presence of 10 mM arabinose. An immunoblot for MAP protein shows that arabinose has no effect on the expression of MAP in the parental EMG-2 strain (Fig. 2B, lanes 3 and 4). However, 10 mM arabinose significantly up-regulates MAP expression (Fig. 2B, lane 1) in the MAPr strain compared to the EMG-2 strain (Fig. 2B, lane 4). Importantly, when the arabinose was slowly depleted from the growth medium of MAPr cultures by multiple dilutions into medium without arabinose, the expression of MAP protein was significantly reduced (Fig. 2B, lane 2). In addition, a decrease in cell divi-

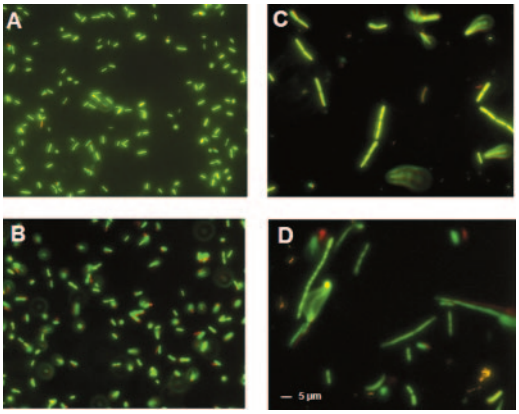

FIG. 3. Morphological changes in *E. coli* due to MAP inhibition by genetic attenuation or chemical inhibition. Parental or MAPr strains were grown in the presence or absence of arabinose and stained with a fluorescence viability stain as described in Materials and Methods. (A) Control, parental strain grown in LB; (B) MAPr strain in LB plus 10 mM arabinose at the 430-min time point in Fig. 2A; (C) parental strain grown in LB plus 500 μg/ml compound C (Table 2); (D) MAPr strain after dilution of arabinose at the 430-min time point in Fig. 2A.

sion was also observed, as judged by optical densities, corresponding to the depletion of arabinose and the loss of MAP expression in the MAPr cultures (Fig. 2A).

Effect of MAP down-regulation on cell survival. To determine whether the decrease in the optical density of these cultures is due to cell death or hampered cell division, bacterial cultures of the MAPr strain grown in medium without arabinose were stained with a fluorescent viability stain. Green fluorescence indicates live cells, whereas red fluorescence is characteristic of dead cells. Only a small number of dead cells were present in cultures from the MAPr strain either before (Fig. 3B) or after MAP attenuation (Fig. 3D); however, enlarged and filamentous cells were detected as a result of MAP attenuation (Fig. 3D), indicating that the slow culture growth was possibly due to inhibition of cell division rather than cell death.

SELDI-TOF MS biomarker profiling. Protein expression was monitored in MAPr versus parental strains by SELDI-TOF MS. Using this method, expression of a subset of proteins under 15 kDa could be simultaneously monitored by enrichment on ProteinChips and analysis by mass spectrometry. A comparison of the protein expression profiles from parental cultures and MAPr cultures showed a mass shift of 131 Da (consistent with an unprocessed N-terminal methionine) in several proteins from the MAPr strain (Table 1). In one example, a pair of small proteins detected at 4,044 Da and 4,060 Da in parental cell lysates (Fig. 4A) were each shifted by 131 Da to 4,175 Da and 4,191 Da, respectively, in the MAPr strain (Fig. 4B). Preincubation of MAPr lysates with recombinant MAP enzyme completely converted these proteins back to the fully processed masses detected in the parental cell lysates (Fig. 4C), thus confirming the attenuation of endogenous MAP activity in the MAPr samples. The other proteins with mass shifts of 131 Da listed in Table 1 were also converted back to fully processed forms upon preincubation with recombinant MAP enzyme (data not shown). Thus, by using the genetically attenuated MAPr strain, eight small proteins were detected and validated as whole-cell biomarkers for diminished MAP activity in whole-cell lysates.

TABLE 1. Whole-cell biomarkers of MAP attenuation with 131-Da mass difference

Peaks detected in MAPr (*m/z*)	Mass difference (Da)	Peaks detected in wild type (*m/z*)
4,175	131	4,044
4,563	131	4,432
6,543	131	6,412
7,911	131	7,780
8,251	131	8,120
9,704	131	9,573
12,782	131	12,651
13,131	131	13,000

Next, this whole-cell assay was applied to a series of in vitro MAP inhibitors using parental EMG-2 cultures. SELDI-TOF MS was used to screen whole-cell lysates for the 131-Da mass shift for each of the eight marker proteins. Twenty different compounds representing 15 compound classes, all of which showed μM to nM inhibitory activity on purified MAP enzyme and MICs under 128 μg/ml, were evaluated for evidence of MAP inhibition in whole bacterial cells. Only 3 of the 15 classes of compounds showed significant inhibition of the MAP enzyme in whole cells as judged by the SELDI-TOF MS readout (Table 2). Two of the three compounds (designated C and J) also represented those compounds with the lowest MIC. Compound C most closely mimicked the profiles of the genetically attenuated (MAPr) strain with significant accumulation of the 131-Da shift in multiple proteins. The growth kinetics associated with this compound were similar to those seen in the MAPr strain after dilutions in medium without arabinose (Fig. 5 compared to Fig. 2A after three dilutions). Furthermore, the morphology for the genetically attenuated bacterial culture (MAPr strain) and the compound C-inhibited parental cells were more elongated and filamentous than control cultures (compare Fig. 3D to C). Finally, the degree to which the culture produced unprocessed biomarker proteins was demonstrated to be dose dependent in that an increased inhibitor concentration resulted in a greater accumulation of the 131-Da shift associated with unprocessed N-terminal methionine (Fig. 6). Thus, the validated, whole-cell, marker proteins allowed 15 compound classes to be rapidly screened to determine which classes produced whole-cell inhibition of the MAP enzyme and thus should be advanced for compound expansion and lead optimization.

DISCUSSION

The current study demonstrates that a protein profiling approach from whole-cell lysates can be used as a screening assay for antibacterial compounds targeted to the MAP enzyme. The ability to screen for activity in whole bacterial cells allowed for the rapid evaluation of 15 compound classes (all of which were good in vitro inhibitors of MAP), and the selection of only 3 to pursue further, thus significantly accelerating the drug discovery process.

One clear advantage of this method is that it allows for a direct measure of the activity of an inhibitor as it acts on the

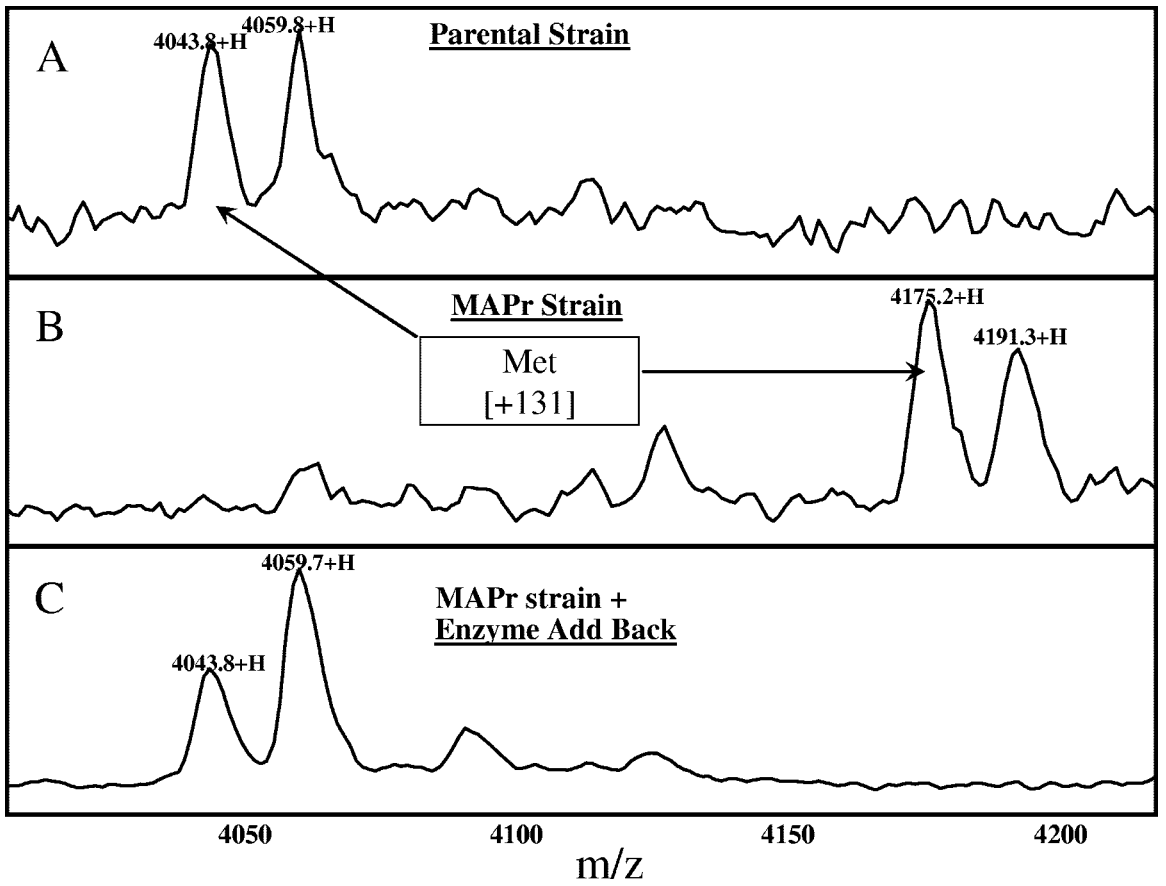

FIG. 4. Biomarkers detected by SELDI-TOF MS. Cultures of parental and MAPr strains after the dilution of arabinose were collected and prepared for SELDI-TOF MS as described in Materials and Methods. Spectra were collected over a range of 3,000 to 15,000 *m/z*, but to demonstrate specific biomarkers, the range from 4,000 to 4,225 *m/z* is presented. (A) Protein peaks detected in the control, parental culture; (B) protein peaks detected in the MAPr culture; (C) protein peaks from the MAPr strain after treatment of the whole-cell lysate with purified recombinant MAP enzyme prior to spotting onto SELDI-TOF ProteinChips.

MAP enzyme in whole cells, thus indicating that the compound gets into the cell and affects growth by inhibition of the target enzyme. As shown in Table 2, even the IC_{50} values in conjunction with MICs did not provide a complete picture for this set of compounds. For example, three compounds (designated C, I, and J) had the lowest MICs measured, but only compounds C and J had a direct effect on MAP activity as judged by the SELDI-TOF MS assay. One explanation for why a compound with a low IC_{50} and MIC (i.e., compound I) does not show MAP inhibition in the intact cell might be that it kills the cells by some other mechanism prior to actually inhibiting the MAP enzyme. Additional studies with this compound to screen for nonspecific membrane disruption in liposomes showed that it had membrane activity consistent with a mechanism of disrupting the physiochemical properties of the membrane (4). Therefore, even a compound with acceptable IC_{50} and MIC data could still have unacceptable side effects that would preclude it from further consideration for advancement. Importantly, this compound was flagged by the SELDI-TOF MS assay as one that does not hit the target enzyme in whole cells. On the other hand, the MIC for compound H was considerable poorer than the other SELDI-TOF MS-positive compounds. This is the expected profile for compounds that have poor cell permeability. Importantly, even with the lower MIC, the fact that inhibition of MAP was detected in whole cells suggests that further expansion of this compound class may be warranted to increase cell permeability. Thus, the SELDI-TOF MS assay, by direct

TABLE 2. Whole-cell screening of MAP inhibitors

Compound	In vitro IC_{50}(μM)	MIC (μg/ml)	Biomarkers detected (*m/z*)
A	13	128	None
B	20	ND[a]	None
C	25	16	4,175, 4,563, 6,543, 12,782
D	25	128	None
E	10	128	None
F	7	128	None
G	18	ND	None
H	1	128	4,175, 4,563, 12,782
I	0.7	16	None
J	22	16	4,175, 4,563, 12,782
K	30	128	None
L	30	128	None
M	4	128	None
N	12	128	None
O	30	128	None

[a] ND, not determined.

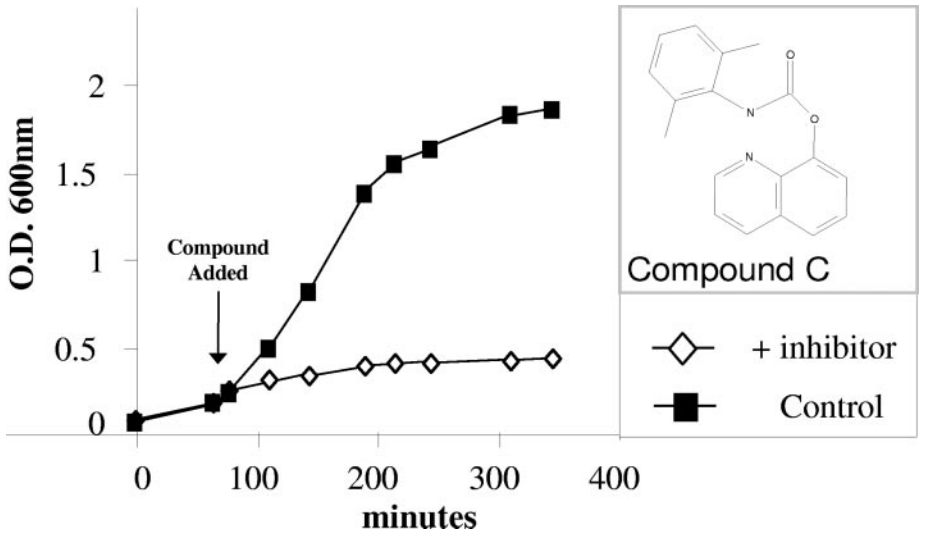

FIG. 5. Growth kinetics of the parental *E. coli* strain in the presence or absence of compound C. Cultures of the parental strain were grown in LB, and at the indicated time, 63 μM compound C was added the culture medium. The growth kinetics were monitored by OD_{600}.

measurement of MAP inhibition in whole cells, provides additional confidence for advancing compound classes that cannot be achieved solely by IC_{50} and MIC measurements.

Upon comparing the SELDI-TOF MS data from the genetically attenuated MAPr cultures with those from parental cultures inhibited with chemical compounds, the similarities in morphology (Fig. 3), growth kinetics (Fig. 2A and 5), and unprocessed Met are demonstrated; however, why are eight biomarkers detected in the MAPr strain after arabinose depletion (Table 1) and only three or four biomarkers detected by compound inhibition (Table 2)? This can be explained by considering what is being measured in each case. Since the

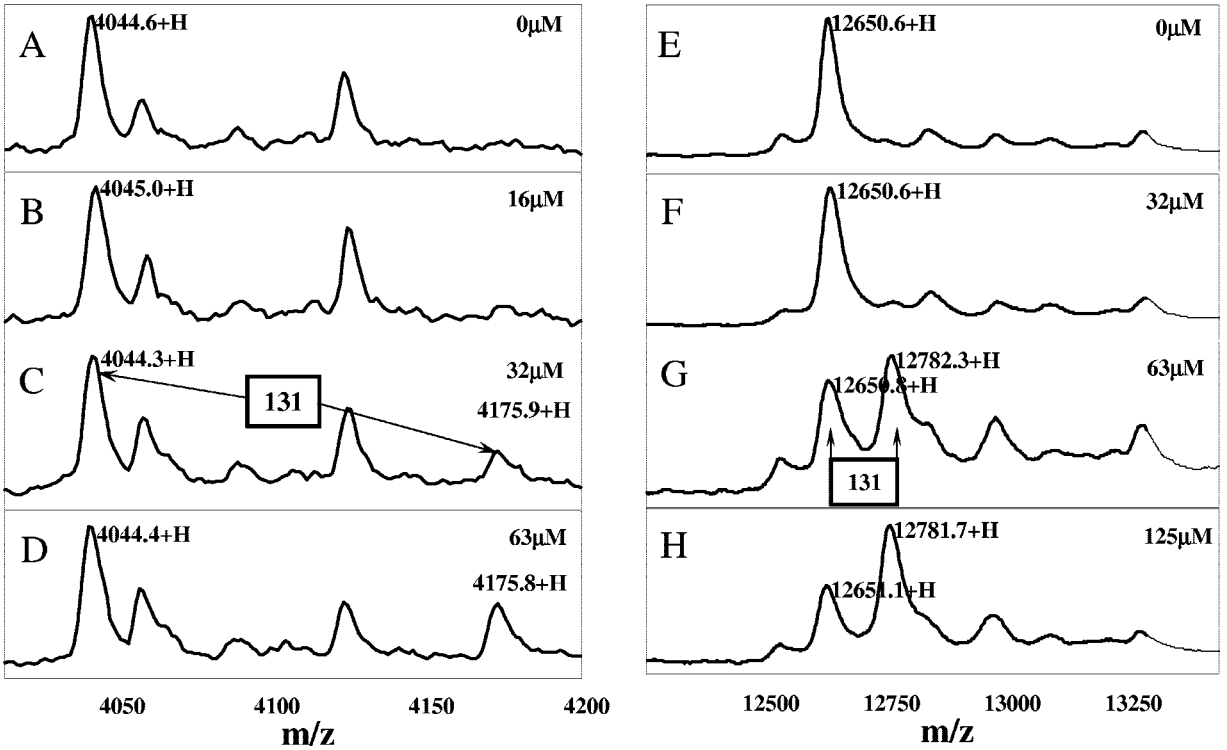

FIG. 6. Dose-dependent detection of unprocessed proteins by SELDI-TOF MS. Parental strain cultures were incubated with increasing concentrations of compound C. Samples were collected at 1-h intervals after the addition of compound C and analyzed by SELDI-TOF MS. (A to D) Dose-dependent increase of unprocessed protein at a *m/z* of 4,176; (E to H) dose-dependent increase of unprocessed protein at a *m/z* of 12,782.

SELDI-TOF MS measurements are made on whole-cell lysates, the proteins detected will be a combination of newly synthesized (and perhaps unprocessed proteins) and the background of existing proteins (fully processed) in the cell. Thus, by the nature of the genetic attenuation protocol to deplete arabinose through three rounds of growth and dilution (Fig. 2A), it is likely that a larger pool of unprocessed proteins will accumulate in the attenuated strain through this process. Upon inhibition with a potent chemical inhibitor, growth arrest can occur more rapidly such that the synthesis and detection of unprocessed proteins may never reach a level that can be sufficiently detected over the existing protein background. This was demonstrated in experiments with compound C and actinonin (an inhibitor of peptide deformalyse), which both arrested cell growth at high concentrations to such an extent that insufficient newly synthesized proteins were produced to detect as unprocessed biomarkers (data not shown; see additional details regarding actinonin and peptide deformylase below). Thus, one important consideration for the success of a SELDI-TOF MS approach (or any biomarker approach that targets essential gene products) is the need to grow cells at sublethal doses of inhibitor in order to capture cells in a state that is meaningful for biomarker detection. Otherwise, one might detect only common markers that are a consequence of cell death rather than the specific mechanism of inhibition.

Beyond the direct benefit for compound screening for MAP inhibitors, two overall themes from this research stand out when considering reapplication for other targets. First, clearly, the use of genetically attenuated strains of *E. coli* to downregulate a target enzyme can provide benefits when evaluating the potency and specificity of inhibitor compounds. This is particularly useful in cases like MAP where no known benchmark compounds were available as a starting point for drug discovery. Using the attenuated strain (MAPr) was also instrumental to our ability to develop and optimize the SELDI-TOF MS assay to measure the effect of MAP attenuation in whole cells. Without the MAPr strain for developing and optimizing the assay, we may have tried a few compound classes without success and given up on the assay. The MAPr strain provided the necessary positive control for assay development. Thus, it is clear that using the genetic attenuation approach would be beneficial for evaluating other bacterial targets.

Second, at the outset, our hypothesis was that we could detect specific biomarkers of MAP inhibition using a global profiling approach on the SELDI-TOF chips. Much to our surprise, we detected eight biomarkers associated with MAP inhibition. Fortunately, these markers arose as a result of inhibiting the MAP enzyme activity, thereby allowing for the direct measurement of the unprocessed, newly synthesized protein. Thus, the SELDI-TOF MS profiling approach should also be useful in evaluating other protein processing targets. To investigate this possibility, we incubated *E. coli* EMG-2 cells with actinonin—a known inhibitor for the bacterial peptide deformylase enzyme (3). Noting that this enzyme is required to remove the formyl group from the initiator *N*-formylmethionine prior to MAP action (Fig. 1) (12), one would expect actinonin treatment to produce a mass shift of 159 Da (28 for the formyl group and 131 for Met). Evaluation of whole-cell lysates at sublethal doses of actinonin produced the expected 159-Da mass shift for many of the same small protein biomarkers as detected for MAP (data not shown), thus demonstrating that the SELDI-TOF MS approach could be applied to whole-cell evaluation of other protein processing targets.

What about profiling for targets without specific protein mass shifts? Clearly, the majority of targets in bacterial systems will not result in a specific and detectible protein mass shift. However, the ability to evaluate protein profiles and detect biomarker peaks in whole-cell lysates that are consistent with hitting a given target may be sufficient to screen compound classes. Given that antibacterial compounds that ultimately result in the arrest of cell growth and/or cell death have a dramatic effect on cellular function, it is likely that various classes of inhibitors (protein synthesis, cell wall, replication, etc.) would produce a unique profile of biomarkers that are diagnostic for that class. As such, one might expect to use a SELDI-TOF MS profiling approach to detect biomarkers for new targets as well. However, in order for this approach to be effective, it is imperative that a specific benchmark inhibitor or genetically attenuated strain of bacteria be evaluated as the positive control. Only then will it be possible to develop sufficient confidence in a biomarker profile to effectively use it in the selection of inhibitor compound classes.

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REFERENCES

1. **Ben-Bassat, A., K. Bauer, S. Y. Chang, K. Myambo, A. Boosman, and S. Chang.** 1987. Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. J. Bacteriol. **169:**751–757.
2. **Chang, S.Y., E. C. McGary, and S. Chang.** 1989. Methionine aminopeptidase gene of *Escherichia coli* is essential for cell growth. J. Bacteriol. **171:**4071–4072.
3. **Chen, D. Z., D. V. Patel, C. J. Hackbarth, W. Wang, G. Dreyer, D. C. Young, P. S. Margolis, C. Wu, Z. J. Ni, J. Trias, R. J. White, and Z. Yuan.** 2000. Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry **39:**1256–1262.
4. **Grinius, L., D. T. Stanton, C. M. Morris, J. M. Howard, and A. W. Curnow.** 2002. Profiling of drugs for membrane activity using liposomes as an *in vitro* model system. Drug Dev. Ind. Pharm. **28:**193–202.
5. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
7. **Layh-Schmitt, G., A. Podtelejnikov, and M. Mann.** 2000. Proteins complexed to the P1 adhesin of Mycoplasma pneumoniae. Microbiology **146:**741–747.
8. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:**6228–6237.
9. **Meinnel, T., Y. Mechulam, and S. Blanquet.** 1993. Methionine as translation start signal: a review of the enzymes of the pathway in Escherichia coli. Biochimie **75:**1061–1075.
10. **Miller, C. G., A. M. Kukral, J. L. Miller, and N. R. Movva.** 1989. *pepM* is an essential gene in *Salmonella typhimurium*. J. Bacteriol. **171:**5215–5217.
11. **Roychoudhury, S., T. Twinem, K. M. Makin, E. McIntosh, B. Ledoussal, and C. Catrenich.** 2001. Activity of non-fluorinated quinolones (NFQs) against quinolone-resistant *Escherichia coli* and *Streptococcus pneumoniae*. J. Antimicrob. Chemother. **48:**29–36.
12. **Solbiati, J., A. Chapman-Smith, J. L. Miller, C. G. Miller, and J. E. Cronan, Jr.** 1999. Processing of the N termini of nascent polypeptide chains requires deformylation prior to methionine removal. J. Mol. Biol. **290:**607–614.
13. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
14. **Vaughan, M. D., P. B. Sampson, and J. F. Honek.** 2002. Methionine in and out of proteins: targets for drug design. Curr. Med. Chem. **9:**385–409.