Evaluation of the Andromas Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry System for Identification of Aerobically Growing Gram-Positive Bacilli

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Matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a rapid and simple microbial identification method. Previous reports using the Biotyper system suggested that this technique requires a preliminary extraction step to identify Gram-positive rods (GPRs), a technical issue that may limit the routine use of this technique to identify pathogenic GPRs in the clinical setting. We tested the accuracy of the MALDI-TOF MS Andromas strategy to identify a set of 659 GPR isolates representing 16 bacterial genera and 72 species by the direct colony method. This bacterial collection included 40 *C. diphtheriae*, 13 *C. pseudotuberculosis*, 19 *C. ulcerans*, and 270 other *Corynebacterium* isolates, 32 *L. monocytogenes* and 24 other *Listeria* isolates, 46 *Nocardia*, 75 *Actinomyces*, 18 *Actinobaculum*, 11 *Propionibacterium acnes*, 18 *Propionibacterium avidum*, 30 *Lactobacillus*, 21 *Bacillus*, 2 *Rhodococcus equi*, 2 *Erysipelothrix rhusiopathiae*, and 38 other GPR isolates, all identified by reference techniques. Totals of 98.5% and 1.2% of non-*Listeria* GPR isolates were identified to the species or genus level, respectively. Except for *L. grayi* isolates that were identified to the species level, all other *Listeria* isolates were identified to the genus level because of highly similar spectra. These data demonstrate that rapid identification of pathogenic GPRs can be obtained without an extraction step by MALDI-TOF mass spectrometry.

A erobically growing Gram-positive rods (GPRs) form a very heterogeneous and extensive group of bacterial species (23). Some of them, such as *Corynebacterium diphtheriae*, *Listeria monocytogenes*, and *Bacillus anthracis* are highly pathogenic. These bacterial species are associated with severe community-acquired infections and may be associated with outbreaks, thus requiring rapid identification for both therapeutic and infection control measures. Other clinically important pathogenic GPR species include *Nocardia* spp. and *Rhodococcus equi*, which can cause severe opportunistic infections. *Corynebacterium urealyticum* and *Corynebacterium jeikeium* are, respectively, associated with urinary tract infections and vascular catheter infections, prosthetic endocarditis, and septicemia and can be multidrug resistant (31, 49). Finally, during the last two decades, a number of species of low pathogenicity, such as *Propionibacterium acnes* and new actinomycetes (*Actinobaculum schaalii*, *Actinomyces neuii*, *Actinomyces turicensis*, and *Actinomyces radingae*), have been associated with various infectious conditions (5, 25, 37). Rapid identification of GPRs is therefore increasingly required for therapeutic and/or epidemiological concerns.

Systematic identification of GPRs is a challenge. Phenotypic identification methods are accurate for identification of pathogenic GPRs. However, it isn't easy to identify most commensal GPR isolates that are frequently cultured in microbiology laboratories by these techniques, due to weak or variable reactivity among single species or because additional tests are required in up to 50% of cases to obtain a 90% correct identification rate (22). In addition, as most recently described GPR species have been char-

acterized using molecular techniques such as 16S rRNA gene sequencing, these species cannot be identified using phenotypic methods in the absence of appropriate identification systems or updated databases (1, 10, 21, 22, 45, 54). In some cases, 16S ribosomal gene sequencing poorly discriminates closely related species, and alternative molecular methods have been developed to solve these issues (28, 36, 38). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a rapid bacterial identification technique that is increasingly used in microbiology laboratories (13, 30, 48). MALDI-TOF MS identification systems are based on the comparison of the tested isolate mass spectrum with reference databases. Several databases and identification strategies have been developed, including the Biotyper (Bruker Daltonics, Bremen, Germany), the Saramis (bioMérieux, Marcy l'Etoile, France), and the Andromas (Paris, France) systems. Previous studies reported that the Bruker MALDI-TOF MS system accurately identified GPR species such as *Listeria* spp., *Nocardia* spp., and *Corynebacterium* species (3, 6, 29,

51). In these studies, a preliminary extraction step was mandatory to obtain satisfactory results, a procedure that is more cumbersome and time-consuming than the direct colony method that can be used to identify Gram-negative bacilli (43). Available MALDI-TOF MS databases have also been evaluated in the routine use of clinical microbiology laboratories, but few GPRs were included, and in some cases, poor identification results were reported (7, 47).

The Andromas identification strategy is based on a limited number of species-specific profiles for each entry (11, 16). A particularity of the Andromas database is that it was built without any extraction step (7, 12). Previous studies have shown that this identification strategy provides good identification of bacteria, mycobacteria, yeasts, and *Aspergillus* spp. by the direct colony method (2, 7, 11, 16, 18, 19, 32). We evaluated the accuracy of this method to identify a large collection of GPR isolates that included both pathogenic and commensal isolates.

MATERIALS AND METHODS

Bacterial isolates. A collection of 659 GPR isolates was set up for this study, originating from French clinical microbiology laboratories and reference centers and from the clinical microbiology laboratory of the University of Buenos Aires, Argentina. Altogether, this collection comprised 16 bacterial genera and 73 bacterial species that are listed in Table 1. Except for *Listeria*, *Nocardia*, *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis*, and *Corynebacterium ulcerans* isolates, we used 16S rRNA gene sequencing as a reference technique to identify clinical isolates. 16S rRNA gene sequences were compared with those of bacterial strains obtained from the GenBank or Bioinformatic Bacterial Identification database (17). A ≥99.0% similarity cutoff was required for definite identification.

Listeria isolates were identified by the French National Reference Centre for *Listeria* with the API Listeria identification system (bioMérieux, Marcy l'Etoile, France) (8, 20, 34) and additional phenotypic confirmation tests (41, 46). All *Nocardia* isolates were identified by the French Reference Center for *Nocardia* using a polyphasic approach, including morphological examination, biochemical profiling, antimicrobial susceptibility testing, and 16S rRNA gene sequencing using specific primers designed to identify *Nocardia* spp. (42). Partial sequencing of the *hsp65* gene was done only for isolates identified as *Nocardia abscessus*, *Nocardia arthritidis*, and *Nocardia beijingensis* to confirm species identification (42). *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* isolates were identified by the French National Reference Centre of Corynebacteria of the *Diphtheriae* Complex using the API Coryne system (bioMérieux, Marcy l'Etoile, France), additional phenotypical assays, and specific molecular methods (a PCR targeting the *dtxR* gene for *C. diphtheriae* and a multiplex PCR targeting the 16S rRNA, *rpoB*, and *pld* genes for *C. pseudotuberculosis* and *C. ulcerans*) (36, 38).

Instrumentation and data analysis. Bacterial isolates were grown on 5% horse blood Columbia agar plates and incubated for 24 h (48 h for slow-growth bacteria) at 37°C in a 10% CO_2 atmosphere. Whole-cell bacteria were deposited without an extraction step using a cotton swab on a MALDI-TOF MS target plate and allowed to dry at room temperature. Samples were then fixed by adding 1 μl of absolute ethanol to inactivate bacteria and allowed to dry at room temperature. Samples were then overlaid with 1 μl of saturated α-cyano-4-hydroxycinnamic acid and allowed to crystallize at room temperature. Samples were processed on the Microflex MALDI-TOF MS spectrometer with the FlexControl software (Bruker Daltonics, Bremen, Germany). Positive ions were extruded at an accelerating voltage of 20 kV in linear mode. Each spectrum was the sum of the ions obtained from 350 laser shots performed automatically on different regions of the same well. The spectra were analyzed in an *m/z* range of 3,500 to 20,000.

Evaluation of the Andromas MALDI-TOF MS system for identification of GPRs. Bacterial isolates were identified using the Andromas software, while being unaware of the identification result of the reference

method. This software compares the mass spectrum of the tested isolate with the spectra included in the Andromas database, taking into account a possible MS peak variation of ±10 *m/z* (7). The Andromas database was created using both reference and clinical isolates routinely used in our laboratory. To date, this database encompasses more than 700 bacterial species, including 33 genera and 156 species of aerobically growing Gram-positive rods. Using this software, species identification is considered to be valid if the percentage of common peaks is ≥68% of those of a species-specific profile of the database. A 10% difference between the first two species diagnostics having the best match in the database is also required to give species identification. If the latter condition is not fulfilled, the identification is considered to be correct at the genus or group level for closely related species if the first two matches belonged to the same genus or group of bacteria. In all other cases, results are considered to be inconclusive.

In this study, a misidentification was defined as a discrepancy between the identification result given by MALDI-TOF MS and the result obtained by 16S rRNA sequencing or other reference identification methods described above. Identifications to the genus level and absence of identification were not considered misidentifications.

Listeria **species discrimination.** A similarity study of *Listeria* isolates' mass spectra was performed using the BioNumerics 5.1 software (Applied Maths, Belgium) for in-gel-view representation and calculation of dendrograms. Similarities between mass spectra were calculated using the Euclidean distance. Hierarchical clustering was performed using the unweighted-pair group method using average linkages (UPGMA) aggregation method.

RESULTS

Except for *Listeria* isolates, 98.5% (594/603) of strains were identified to the species level, and only 1.2% (7/603) of them could not be identified beyond the genus level (Table 1). Correct identifications were obtained for the closely related pathogenic species *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*, as well as for frequently encountered nonlipophilic commensal *Corynebacterium* species that are difficult to identify using biochemical tests: *C. striatum* or *C. amycolatum*, which have been misidentified as *C. xerosis* (21); *C. simulans*, which fits the biochemical profile of *C. striatum* or *C. minutissimum* (53), and *C. aurimucosum*, which is difficult to differentiate from *C. minutissimum* (55). MALDI-TOF MS also allowed a rapid identification of bacterial species that would have required molecular methods for definitive identification as *Nocardia* spp., *Rhodococcus equi*, *Erysipelothrix rhusiopathiae*, *Actinomyces* spp., and *Bacillus* and *Lactobacillus* spp. (9). In most cases, MALDI-TOF MS performed as well as extended phenotypic and specific molecular methods to identify GPR isolates.

Conversely, except for *Listeria grayi* isolates that were identified to the species level, all other *Listeria* species isolates could not be identified beyond the genus level. Indeed, the mass spectrum of these isolates always matched more than one *Listeria* species-specific profile of the database with a high similarity value (≥68% of common peaks). Moreover, the 10% difference between the two best matches that is required by the Andromas algorithm to provide species identification was not reached. Therefore, identification was only given at the genus level. The similarity analysis of the mass spectra of *Listeria* isolates accounts for these results, showing that *Listeria grayi* and non-*grayi* isolates form two distinct clusters with high intrinsic similarity values (≥70%), but these two clusters only share a 40% similarity value (Fig. 1). These findings corroborate molecular 16S and 23S rRNA phylogenetic studies that

TABLE 1 Identification of 659 Gram-positive rods using the MALDI-TOF MS Andromas system[a]

Genus or species	No. of isolates tested	Identification result (no. of isolates)			
		Species level	Genus level	Not identified	Error
Actinobaculum spp.[b]	18	18	0	0	0
A. schaalii	14	14	0	0	0
A. massiliense	4	4	0	0	0
Actinomyces spp.[b]	76	75	1	0	0
A. europaeus	1	1	0	0	0
A. funkei	4	4	0	0	0
A. naeslundii	1	1	0	0	0
A. neuii		33	33	0	0
A. odontolyticus	5	5	0	0	0
A. radingae	6	6	0	0	0
A. turicensis	23	23	0	0	0
A. urogenitalis	2	2	0	0	0
A. viscosus	1	0	1	0	0
Bacillus spp.[b]	21	21	0	0	0
B. cereus/B. thuringiensis	10	10	0	0	0
B. licheniformis	2	2	0	0	0
B. pumilus/B. safensis	4	4	0	0	0
B. subtilis	3	3	0	0	0
B. simplex	2	2	0	0	0
Corynebacterium spp.[b]	342	338	2	1	1
C. amycolatum	97	97	0	0	0
C. aurimucosum	30	27	2	0	1
C. coyleae	3	3	0	0	0
C. diphtheriae	40	39	0	1	0
C. glucuronolyticum	10	10	0	0	0
C. imitans	2	2	0	0	0
C. jeikeium	16	16	0	0	0
C. massiliensis	1	1	0	0	0
C. pseudodiphtheriticum	10	10	0	0	0
C. pseudotuberculosis	13	13	0	0	0
C. simulans	25	25	0	0	0
C. singulare	1	1	0	0	0
C. striatum	44	44	0	0	0
C. tuberculostearicum	18	18	0	0	0
C. ulcerans	19	19	0	0	0
C. urealyticum	10	10	0	0	0
Lactobacillus spp.[b]	30	30	0	0	0
L. casei		1	1	0	0
L. crispatus	8	8	0	0	0
L. delbrueckii	1	1	0	0	0
L. fermentum	2	2	0	0	0
L. gasseri	7	7	0	0	0
L. jensenii	6	6	0	0	0
L. rhamnosus	5	5	0	0	0
Listeria spp.[b]	56	4	52	0	0
L. grayi		4	4	0	0
L. innocua	5	0	5	0	0
L. ivanovii	5	0	5	0	0
L. monocytogenes	32	0	32	0	0
L. seeligeri	5	0	5	0	0
L. welshimeri	5	0	5	0	0

TABLE 1 (Continued)

Genus or species	No. of isolates tested	Identification result (no. of isolates)			
		Species level	Genus level	Not identified	Error
Nocardia spp.[b]	46	42	4	0	0
N. abscessus	4	4	0	0	0
N. asteroides	2	2	0	0	0
N. arthritidis	1	0	1	0	0
N. beijingensis	3	3	0	0	0
N. brasiliensis	7	6	1	0	0
N. cyriacigeorgica	7	6	1	0	0
N. farcinica	11	10	1	0	0
N. nova		5	5	0	0
N. mexicana	1	1	0	0	0
N. otitidiscavium	2	2	0	0	0
N. paucivorans	1	1	0	0	0
N. veterana	2	2	0	0	0
Propionibacterium spp.[b]	29	29	0	0	0
P. acnes	11	11	0	0	0
P. avidum	18	18	0	0	0
Other GPR species[c]	41	41	0	0	0
Arthrobacter cumminsii	1	1	0	0	0
Brevibacterium casei	3	3	0	0	0
Brevibacterium paucivorans	1	1	0	0	0
Dermabacter hominis	12	12	0	0	0
Erysipelothrix rhusiopathiae	2	2	0	0	0
Gardnerella vaginalis	3	3	0	0	0
Paenibacillus motobuensis	1	1	0	0	0
Rhodococcus equi	2	2	0	0	0
Turicella otitidis	16	16	0	0	0

[a] The mass spectra of the isolates studied were compared with those of the whole Andromas database, which encompasses more than 700 bacterial species, including 33 genera and 156 species of aerobically growing Gram-positive rods (GPRs).
[b] For these genera, the total numbers of taxa included in the Andromas database are 18 *Actinomyces*, 3 *Actinobaculum*, 13 *Bacillus*, 32 *Corynebacterium*, 24 *Lactobacillus*, 6 *Listeria*, 25 *Nocardia*, and 4 *Propionibacterium* species.
[c] For these genera, the total numbers of taxa included in the Andromas database are 5 *Arthrobacter*, 6 *Brevibacterium*, 1 *Dermabacter*, 1 *Erysipelothrix*, 1 *Gardnerella*, and 3 *Paenibacillus* species, 1 *Rhodococcus* species, and 1 *Turicella* species.

showed that two clusters can be identified within this genus: one for *L. grayi* and a second for other *Listeria* species (14, 44).

Only 7 other GPRs were identified to the genus level, including 4 *Nocardia* and 2 *C. aurimucosum* isolates and 1 *A. viscosus* isolate. The 4 *Nocardia* isolates, which belonged to 4 different species, were not identified to the species level because of poor mass spectra, a technical issue that has been previously reported for *Nocardia* spp. without an extraction step (51). The two *C. aurimucosum* isolates and the *A. viscosus* isolate had satisfactory mass spectra but matched another species-specific profile of the same genus with a less than 10% difference.

Finally, 1 *C. aurimucosum* isolate was misidentified as *C. striatum*, and 1 *C. diphtheriae* isolate could not be identified. Analysis of the mass spectrum of the *C. aurimucosum* isolate showed that it corresponded to a new specific *C. aurimucosum* profile. The *C. diphtheriae* isolate was not identified because its mass spectrum comprised only 5 peaks, whereas species-specific profiles of the
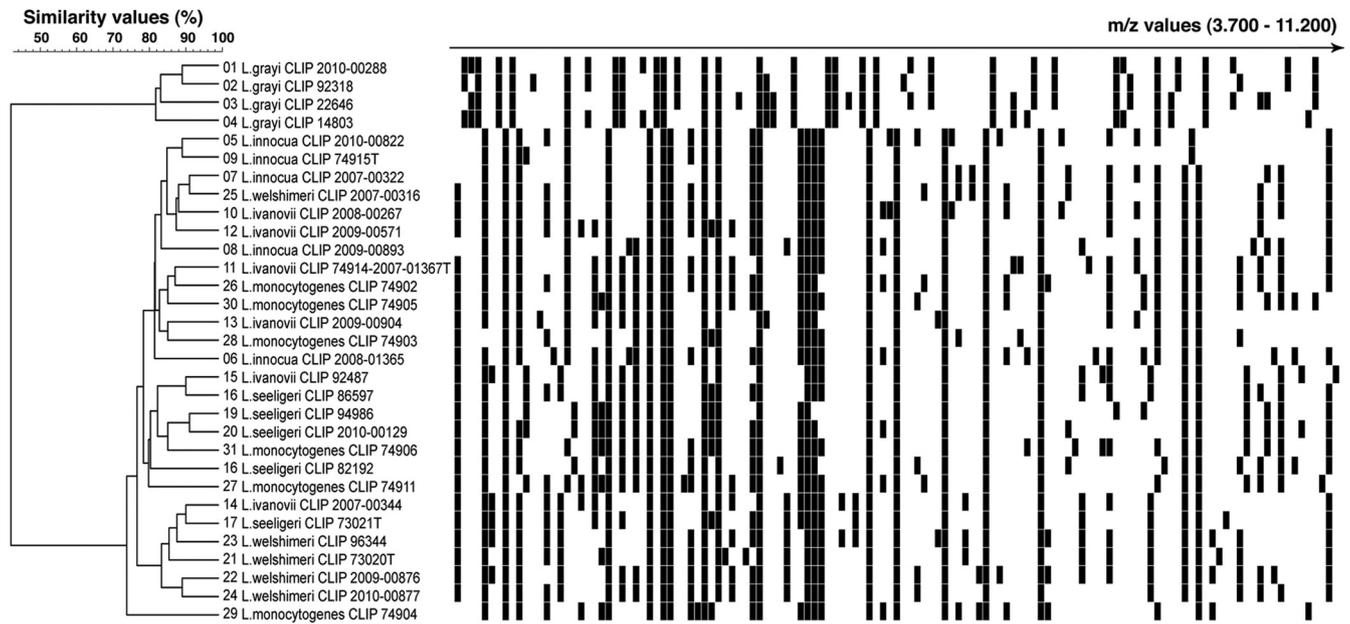
FIG 1 MALDI-TOF MS discrimination of *Listeria* species. MALDI-TOF MS profiles of *Listeria* isolates, as obtained by the direct colony method, are visualized in a gel-view representation. Spectra were clustered using the UPGMA clustering algorithm. The scale represents the percentage of matching peak mass signals between individual spectra.

closely related species *C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans* comprise a minimum of 23 peaks. In that case, a formic acid protein extraction step slightly improved the mass spectrum profile (13 peaks), but species identification could not be obtained because the two best matches in the database were too close (*C. diphtheriae* at 63% and *C. pseudotuberculosis* at 60%). This non-toxigenic isolate was recovered from a patient with bacteremia. It grew well on agar plates and was correctly identified by phenotypic and molecular assays.

All *Bacillus cereus* isolates were identified as *Bacillus cereus*/*B. thurigiensis* group since these two species display similar species-specific spectra in the Andromas database. Four additional *Bacillus* isolates were identified as *B. pumilus*/*B. safensis* for the same reason and because they cannot be identified to the species level using 16S rRNA gene sequencing.

DISCUSSION

This work is the first MALDI-TOF MS study that addressed the issue of the accuracy of this new bacterial identification method against a fairly extensive collection of Gram-positive rods, including both pathogenic and commensal species. The most important result of our study is that the vast majority of pathogenic GPRs could be identified to the species level. In addition, we also accurately identified nonpathogenic or low-pathogenicity species. As most of these strains were obtained from clinical microbiology laboratories and represent the GPRs that are most frequently recovered in this setting, our data suggest that this identification strategy can also be used to rule out pathogenic pathogens and to describe new GPR-associated infectious conditions.

In this study, MALDI-TOF MS identification of bacterial isolates were obtained by the direct colony method—i.e., under workflow conditions that can be routinely used in all microbiology laboratories. Similar results were only obtained after a preliminary extraction step using the Bruker Biotyper system (3, 6, 29,

51). Each MALDI-TOF MS identification system has its own specificities, including the hardware itself (the mass spectrometer), the way the database was built, and the algorithms used to compare the mass spectrum of a sample with those of the database. Therefore, it is not an easy task to identify the reason why better results were obtained with the Andromas system by the direct colony method. It is well established that the quality of mass spectra varies according to bacterial genera. For instance, better MALDI-TOF MS acquisitions (i.e., higher number of peaks and/or higher relative intensity of peaks) are usually obtained for Gram-negative bacteria (except for mucoid colonies) compared to enterococci and Gram-positive rods (4). Consequently, using a MALDI-TOF database that was built with an extraction step, it is likely that identification errors will occur more frequently for Gram-positive bacteria than for Gram-negative bacteria. The Andromas database was built without any extraction step, a technical issue that probably had an important impact on the quality of results we obtained. To the best of our knowledge, the way the bioMérieux and Bruker databases were engineered has not been previously reported or published.

Our data suggest that species identification of *Listeria* spp. by the direct colony method can only be achieved at the genus level, except for *L. grayi*. It has been reported that species identification and typing of *Listeria* spp. can be obtained with the Biotyper system (6). Nevertheless, a complex extraction step and several centrifugations that may not be routinely implemented in clinical microbiology laboratories were required to obtain these results. As *L. monocytogenes* causes almost all cases of human listeriosis, rapid identification of a GPR isolate as *Listeria* sp. is significant progress. For a limited number of GPRs belonging to other genera, no identification was obtained at the species level, because the mass spectrum of the tested isolate matched two species-specific profiles of the same genus, because of an atypical mass spectrum, or due to poor mass spectrum acquisition. Altogether, these ob-

servations illustrate the limitations, albeit limited, of the Andromas MALDI-TOF MS strategy for identification of GPRs. By the direct colony method, we obtained species identification rates of *Corynebacterium* and *Nocardia* isolates similar to those reported by the extraction method using the Biotyper system, suggesting that a simpler approach can be used to identify GPRs.

Rapid identification of GPRs should have an important clinical impact on the diagnosis of GPR-associated infectious diseases. Rarely encountered diseases, such as granulomatous lymphadenitis due to *C. pseudotuberculosis* or cutaneous manifestations of *C. ulcerans* or *C. diphtheriae*, may be easier to diagnose (27, 33, 52). In that case, the main advantage of this direct colony method is to give rapid identification of colonies that cannot be distinguished from those of other nonlipophilic *Corynebacterium* species. MALDI-TOF MS should also improve the diagnosis of fastidious or recently described GPR species, like new *Actinomyces* species (26, 35, 39, 45). In addition, rapid identification of GPRs should improve the probabilistic antimicrobial treatment of patients. Indeed, some GPRs have specific and atypical susceptibility patterns, such as resistance to broad-spectrum cephalosporins for *L. monocytogenes* and resistance to glycopeptides for *E. rhusiopathiae*, some *Nocardia* spp., and some *Lactobacillus* spp. (15, 24, 50). Some GPRs, such as *C. jeikeium*, and, to a lesser extent, *C. urealyticum* and *C. amycolatum*, can be multidrug resistant (40).

To conclude, our data suggest that the Andromas strategy is an accurate method for identification and/or screening of pathogenic GPRs. As taxonomic changes and characterization of new species or genomospecies of GPRs are frequent, continuous enrichment of the MALDI-TOF MS databases will have to be performed as a dynamic process.

ACKNOWLEDGMENTS

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