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USGA Executive Summary

Donald Y. Kobayashi and Bruce Clarke. Identification of parasitic bacteria as biological control agents against summer patch disease. Rutgers University, Department of Plant Pathology, new Brunswick, NJ 08903.

Executive Summary

Stenotrophomonas (*Xanthomonas*) *maltophilia* 34S1 (Sm34S1) was previously identified as a biological control agent capable of controlling summer patch disease, caused by *Magnaporthe poae*. Greenhouse/growth chamber studies indicated that Sm34S1 reduced foliar symptoms on Kentucky bluegrass by as much as 70% compared to untreated disease controls. When Sm34S1 was applied to plants on a repeated basis, summer patch was suppressed at high, sustained levels. Colonization studies suggested that Sm34S1 populations should be established within the turfgrass rhizosphere at levels above 10^7 cfu/g sample during a two week period, and should remain above 10^5 cfu/g sample to achieve effective control. Sm34S1 was applied to pathogen-inoculated field plots located in a three year old Kentucky bluegrass stand that received minimal maintenance during the summer of 1995. Summer patch symptom development was not suppressed by Sm34S1 during that year. Population studies indicated that Sm34S1 was maintained at levels between 10^4 and 10^7 cfu/g sample. Sm34S1 was applied to pathogen-inoculated field plots in 1996 consisting of annual bluegrass/bentgrass green. Summer patch symptoms did not develop in field plots during 1996. Studies indicated that Sm34S1 populations fluctuated in the turfgrass rhizosphere over a range greater than that observed in 1995; however, on occasion, populations were established above the critically determined level of 10^7 cfu/g sample.

A single chitinase gene was cloned from Sm34S1 and the nucleotide sequence was determined. The gene encoded a single polypeptide of ca. 1.6 kb, and was associated with a protein of 51.1 kdal in size. Site directed mutagenesis of the gene in 34S1 resulted in loss of chitinase activity, and a significant reduction in biocontrol of summer patch by this organism. Chitinase activity and biocontrol of summer patch was restored when the cloned gene was reintroduced into the mutant. Studies indicated that chitinase was expressed under conditions of nutrient stress and in the presence of chitin. These studies provide strong evidence for the role of chitinase in biocontrol activity by 34S1, and information towards understanding the conditions in which the gene is expressed.

Previously isolated biocontrol strains that appeared similar to *S. maltophilia* 34S1 were compared on a taxonomic basis. Fatty acid analysis (MIDI) and nutritional utilization (Biolog) suggested that two isolates, N4-7 and N4-15, previously recovered from the turfgrass rhizosphere and demonstrated to have summer patch suppressive abilities, were closely related *Stenotrophomonas*, *Xanthomonas* and *Xylella* species. Serological tests using polyclonal antibodies made against N4-7 indicated relatedness to *Xylella* and N4-15, but not to *Stenotrophomonas*. Comparisons of 16s rDNA sequences confirmed the relatedness of both N4-7 and N4-15 to *Xylella* and *Stenotrophomonas*. However, N4-7 appeared most closely related to an unidentified, hydrothermal vent eubacterium. These observations provide further information towards understanding the composition and diversity within the turfgrass microbial ecosystem.

Introduction

Summer patch disease is caused by the ectotrophic, root-infecting fungus, *Magnaporthe poae*. The disease is extremely damaging to turfgrass, affecting cool-season varieties under conditions of high soil temperature and high water potential. Disease development is enhanced by conditions that contribute to turfgrass root stress, such as low mowing heights or compacted soil. Symptoms of the disease are observed as patches of foliar necrotic areas, which will coalesce under severe disease conditions. In addition to aesthetic problems, summer patch is also problematic due to its damaging effect in playability on recreation turf. These problems become of greater economical importance when diseased areas are rapidly colonized by resistant, less desirable species of grass or weeds.

Demands for high quality turf results in control measures for summer patch and other diseases that rely heavily on regular applications of fungicides. Our primary objective is to investigate the use of beneficial bacteria for control of summer patch and other diseases of turfgrass. The use of biological methods for disease control on turfgrass can be envisioned as a component of integrated strategies to maintain high quality turfgrass, and reduce the reliance on chemicals for disease control.

The primary research focus during 1996 was to investigate repeated application of biocontrol bacteria on highly maintained turfgrass greens inoculated with *Magnaporthe poae,* to determine the mechanism of biocontrol in our agents to optimize conditions for biocontrol, and to clarify the taxonomic identity of biocontrol agents.

RESULTS

Application of Sm34S1 to field plots consisting of annual bluegrass greens during the summer of 1996. *Stenotrophomonas maltophilia* Sm34S1 was applied to field plots during the summer months of 1996. The field plot consisted of 40, 3'x3' squares on an annual bluegrass/bentgrass green (80:20) that was maintained at the standard conditions of 5/23 inches, cut 3-4 times a week and irrigated 2-3 times a week. (General maintenance of the green throughout the year also included top dressing with sand 3-4 times a year, and aerification during the fall and spring of each year.) Four sites within each plot were inoculated with *Magnaporthe poae* at the corners of a 1.5' square placed in the center of the plot. Pathogen inoculum consisted of sterile oat seeds colonized with *M. poae*, which was generated by first sterilizing oat seeds, and then inoculating the seeds with fungus previously grown on potato dextrose agar. Two sites were inoculated with 60 cm^3 and two sites with 20 cm^3 of infested oat seeds. Four treatments, replicated 10 times each, were used in the experiment. The four treatments consisted of the following:

1. Sm34S1 applied on a weekly basis
2. Sm34S1 applied on a biweekly basis
3. *M. poae*-inoculated disease control
4. Uninoculated disease control.

Plots were inoculated on 11 June, and the first treatments were applied on the 12 June. Treatment applications continued weekly until the 4 Sep. Bacterial treatments were generated by growing cells in a rich medium over night, pelleting by centrifugation, and resuspending to a concentration of $5x10^8$ cfu/ml. One liter of this solution was then diluted with 2 l of H_2O (3 l total, which was previously determined as the volume that saturated the soil), and was evenly applied to a plot using a hand-held watering can. 3 l of H_2O was applied to *M. poae*-inoculated disease control plots. During the weekly applications, all plots not scheduled to receive bacterial treatments were treated with the application of 3 l of water.

Core turf samples were collected from randomly selected sites within 5 plots that represented each bacterial treatment. Samples were collected before and after each application. For the biweekly treatment, samples were also collected once during each week that bacteria were not applied to the plot. After samples were collected, the holes left by removed cores were filled with standard top dressing soil. Each core was separated into soil and rhizosphere (consisting of roots with loosely adhering soil) samples, and was dilution plated to enumerate bacterial populations. Sm34S1 is naturally resistant to the antibiotic rifampicin. Therefore, rifampicin was added to tryptic soy broth agar to specifically select for growth of Sm34S1.

Typical summer patch symptoms were not produced on the inoculated turfgrass greens during 1996. There are a few reasons that are thought to contribute to the lack of symptom production in 1996. Unusually cool summer weather similar to that in 1993 most likely contributed to the lack of symptom development (summer patch symptoms also did not occur in field trials conducted in 1993). However, a second reason is that, although the pathogen strain used to inoculate plots was originally isolated from annual bluegrass, field trials to date have been conducted on Kentucky bluegrass. Consequently, it is possible that the turf green used in this study, which consisted of a mix of annual bluegrass and bentgrass, may also have affected symptom production.

Despite the lack of summer patch symptom development, populations of Sm34S1 were successfully monitored throughout the 1996 summer season. Results of this field experiment indicated that the bacterium in both the rhizosphere and soil of the highly maintained turf green comprised of annual bluegrass/bentgrass was similar to populations observed on Kentucky bluegrass stands, which were not as highly maintained (Fig. 1). In general, populations spanned a greater range in 1996, ranging from $>10^3$ to $<10^8$ cfu/g rhizosphere sample, compared to 1995, which ranged between $>10^4$ to $<10^7$ cfu. On more than one occasion, populations of Sm34S1 did reach levels greater than 10^7 cfu/g sample in 1996, a level that was determined critical by greenhouse/growth chamber studies (Fig. 2). However, populations often decreased below 10^5 cfu/g sample, which appears to be the critical minimum level established in greenhouse/growth chamber studies (Fig. 2). In contrast, soil populations in were generally greater in 1995 than in 1996. Reasons for these observations are unclear, however, conditions for survivability may have favored Sm34S1 in 1995 compared to 1996. Based on these observations, it is apparent that higher initial concentrations of bacteria which are applied to turf are necessary to reach populations levels critical for disease control. It is evident that highly maintained turfgrass may slightly, but does not significantly, improve populations of Sm34S1 in the rhizosphere of turfgrass.

Establishment of the role of chitinase in biocontrol of summer patch by Sm34S1, and induction of its expression. A single chitinase gene was cloned from Sm34S1, and molecularly characterized. A single open reading frame of ca. 1.6 kb was identified, which encoded for a predicted protein of ca. 90 kdal. Expression of the gene in *Burkholderia cepacia* M53 produced a protein product of ca. 88 kdal in size, in good agreement with the predicted size of 90 kdal. This protein was associated with chitinase activity, as detected by clearing of colloidal chitin in agar, and the production of fluorescence when cell culture filtrates were incubated with the substrate 4-methylumbelliferyl-β-D-*N,N'*-diacetylchitobioside and 4-methylumbelliferyl-β-D-*N,N'*-triacetylchitotriose. In Sm34S1, chitinase activity was associated with a protein of 51.1 kdal in size. The smaller size compared to the predicted size and the size observed by the protein produced by *B. cepacia* M53 is suggestive of post-translational processing. Site-directed mutagenesis of the gene in Sm34S1, resulted in strain C5. This mutant isolate lacked chitinase activity, and no longer produced the protein of 51.1 kdal previously associated with chitinase activity. Furthermore, the isolate was significantly reduced in biocontrol ability (Fig. 3). Mobilization of the cloned gene into the mutant restored chitinase activity, and restored production of the 51.1 kdal protein. In biocontrol assays, summer patch suppression was significantly reduced when treated with the chitinase mutant C5 compared to Sm34S1. Mobilization of the cloned gene into mutant C5 restored its ability to suppress summer patch at levels comparable to Sm34S1 (data not shown).

These observations provide strong evidence that chitinase production by Sm34S1 plays a major role in biocontrol of summer patch. Consequently, characterization of the expression of chitinase is critical to determining optimal conditions for biocontrol. Initial characterization of the chitinase gene Beginning with a 3 kb *Xho*I-*Sal*I fragment, a transcriptional fusion of a promoterless *uidA* gene (GUS) was fused to a *Bam*HI site located internal to the open reading frame encoding the chitinase gene, and was cloned into the broad host range vector, pRK415. The construct was mobilized into Sm34S1, and tested on various media to study its expression. Differential expression of GUS was observed, by the appearance of a chromophore, when the isolate was plated on various nutrient media containing various supplementations to the media. Expression was high when cells were plated on a minimal medium, as compared to relatively no expression when plated on a nutrient rich medium. These observations indicated that chitinase production in induced under conditions of nutrient stress. Chitinase production can be induced to higher levels when chitin is added to any media. This observation indicates that the substrate induces expression of the gene. Studies are still preliminary, and exact specific environmental conditions/signals responsible for expression of the gene, as well as the coordinate expression with other extracellular enzymes, are still unclear.

Taxonomic characterization of selected biocontrol bacteria. Based on our observations of isolating potential pathogen antagonists, the bacterial species *Stenotrophomonas maltophilia* and closely related organisms appear to comprise a significant portion of the microbial diversity within the turfgrass rhizosphere. Generally speaking, however, little is known about the microbial composition or diversity within the turfgrass rhizosphere. At least three isolates that express good biocontrol against summer patch, N4-7, N4-15 and Sm34S1, appear to be closely related. Their taxonomic relationship, however, remains somewhat unclear. To date, Sm34S1 is the only isolate that has been identified to the species level by standard, commercial identification methods.

These include Biolog, the chemical redox identification system of Microlog, Inc., and the fatty acid composition analysis system of Microbial Identifications, Inc. Biochemical and morphological comparisons, along with the commercial identification methods, indicated that N4-7 and N4-15 were closely related to Sm34S1, but did not key out taxonomically to *Stenotrophomonas maltophilia*. In molecular characterizations, both N4-7 and N4-15 produced β-1,3-glucanase activity, an enzyme like chitinase that is important in fungal degradation. In contrast, Sm34S1 is devoid of this enzyme activity. To further understand the taxonomic relationship of N4-7 and N4-15 to Sm34S1, comparisons of 16S Rana sequences were conducted. Results from searching databases indicated that N4-7 is related to members of the genera *Stenotrophomonas*, *Xanthomonas*, and *Xylella*, but is most closely related to an unidentified hydrothermal vent eubacterium. Serological comparisons using polyclonal antibodies made to N4-7 indicated a serological relatedness to *Xylella fastidiosa*, but not to *S. maltophilia*. 16s Rana sequence phylogenetically positioned N4-15 between N4-7 and *S. maltophilia*. These results suggest that N4-7 is a new bacterial genus, which may belong to a group of several related bacteria that include isolates such as N4-15 and Sm34S1, which contribute to the diversity and competitive antagonism of microbes within the turfgrass rhizosphere.

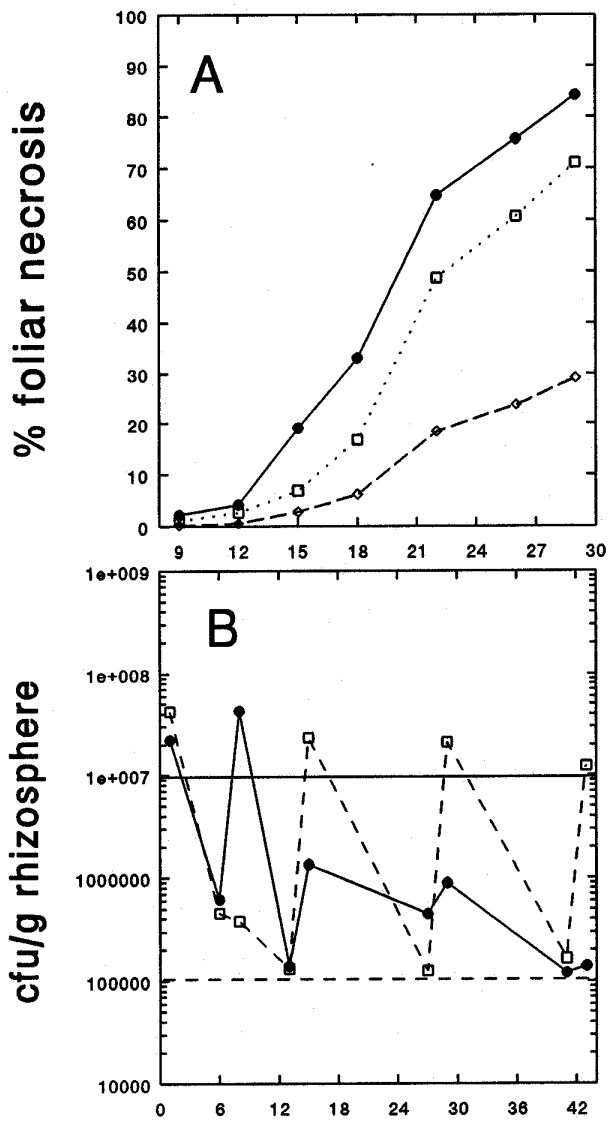

Figure 2. Summer patch suppression on Kentucky bluegrass and rhizosphere populations by repeated applications of *Stenotrophomonas maltophilia* 34S1 (Sm34S1) in greenhouse/growth chamber studies. **A.** Foliar necrosis of summer patch symptoms in untreated controls (solid line with closed circles), standard application of Sm34S1 (dotted line with open squares), and repeated application of Sm34S1 on a 2 week schedule (dashed line with open diamond). X axis represents days in growth chamber. **B.** Populations of Sm34S1in the rhizosphere of Kentucky bluegrass after standard application (solid line with closed circles) and after applications repeated on a 2 week schedule (dashed line with open box). X axis represents days after first application. The solid horizontal line represents predicted required population levels, based on levels reached by repeated applications of Sm34S1. The dashed horizontal line represents the predicted minimum population level that Sm34S1cannot decrease below to maintain disease control.

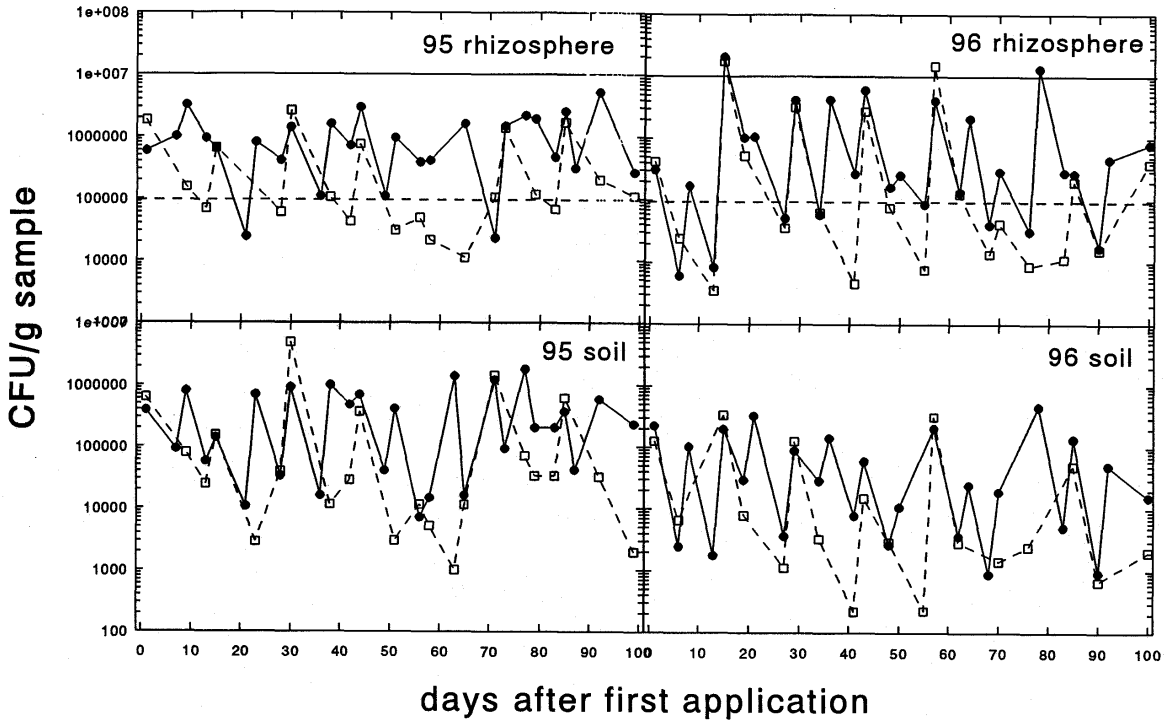

Figure 1. Populations of *Stenotrophomonas maltophilia* Sm34S1 in the turfgrass rhizosphere and soil in field experiments conducted in 1995 and 1996. The experiment in 1995 was conducted on a Kentucky bluegrass stand. The experiment in 1996 was conducted on an annual bluegrass/bentgrass green. Solid lines with closed circles represent populations of Sm34S1 applied to plots on a weekly basis. Dashed lines with open squares represent Sm34S1 applied to plots on a biweekly basis. The solid horizontal lines in rhizosphere graphs represent the level above which populations should be established and the dashed horizontal lines represent critical minimum levels which populations should not drop below based on greenhouse/growth chamber studies.

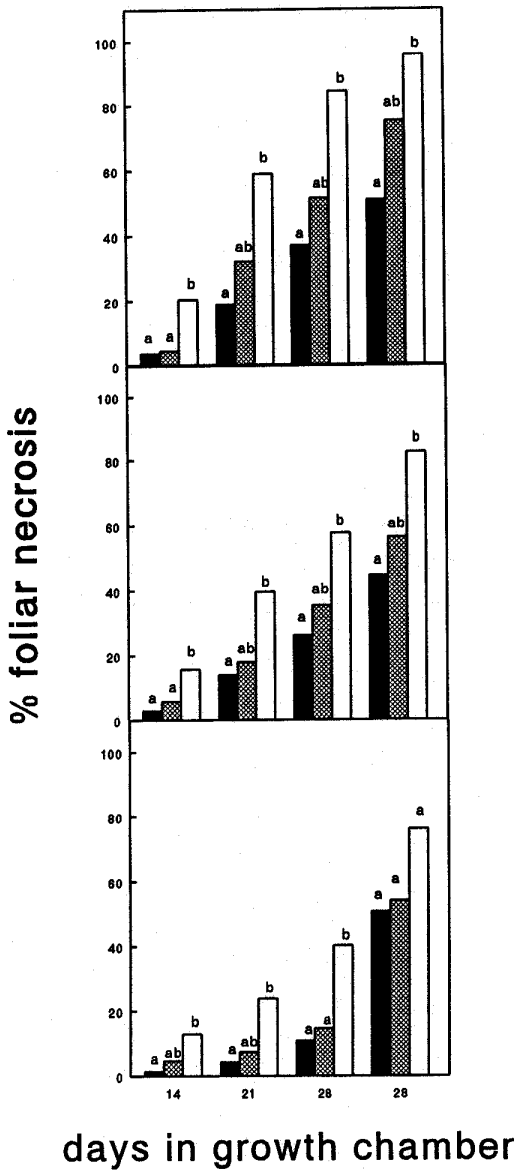

Fig. 3. Symptom development of summer patch disease on Kentucky bluegrass treated with *Stenotrophomonas maltophilia* 34S1 (Sm34S1). A, B, and C represent three separate trials. Experiments were conducted in containers in the growth chamber. Values, representing the percentage foliar necrosis, are the means of 10 replications. Closed bars, Sm34S1; hatched bars, *S. maltophilia* C5 (chitinase mutant of Sm34S1); open bars, untreated control plants. Bars with similar letters within a specific date are not significantly different according to Duncan's multiple range test.