ELIZABETH NOLAN: We're going to end the unit on synthesis today. And the focus of today's lecture will really be looking at one system in detail and the types of experiments that are done to elucidate a biosynthetic pathway for a non-ribosomal peptide. And so just to recap from last time, if we think about studying assembly lines in lab, and we're thinking about this for a non-ribosomal peptide synthetase, what needs to be done?

So first, it's necessary to, typically, overexpress and purify domains, didomains or modules. And so on Monday, it came up that often, these proteins are enormous and it's not possible or feasible to express entire modules, or entire proteins that have multiple modules. So oftentimes, people will look at individual domains, or didomains, which are smaller and more amenable, to overexpression in an organism like E. coli.

Then it's necessary to assay for A domain activity. So we're called the A domains through the adenylation domains. And the question is, what monomer is selected and activated? And so the ATP-PPi exchange assay comes up here. There needs to be assays for loading of the T domain, or carrier protein, with the monomer. Assay for peptide bond formation, which is the condensation domain. And then often, some assay for chain released by the thioesterase domain. OK, so assay for TE activity. Chain release.

And so in terms about of thinking about these T domains, we learned that these T domains need to be post-translationally modified with the Ppant arm, which means we need an enzyme called a PPTase. And so in many cases, we don't know what the PPTase is for a given gene cluster. And what's done, often in the lab, is that a PPTase from B. subtilis, named Sfp, is used in order to post-translationally modify the T domains with the Ppant arm. So there is a serine residue in these T domains that gets modified. We looked over that in a prior lecture.

So this one is very useful. And if you don't know the enzyme to use, people will use recombinant Sfp And just recall, we have the T domain. There's a serine moiety. We have a PPTase. That's going to stick on the P pant arm here. So we call this apo, holo, and then the

amino acid, or aryl acid monomer, in the case of NRPS gets loaded here via a thioester.

And so Sfp can be used to get us here. And even what people have done is make modified analogs, where there's some R group. So you can imagine using chemical synthesis to load a monomer, or even some other type of group that, for some reason, you might want to transfer here. And this Sfp is very promiscuous and it can do that.

And so the take-home here is if you need a PPTase, overexpress, purify, and utilize Sfp. Here's just an example for review, where we have a carrier protein, so a T domain, and we have the PPTase activity here, Sfp, attaching this Ppant arm. And here, it's described with an R group. And just to give you an example of possibilities here, there have been many reports of CoA analogs being transferred to T domains by Sfp. And these can range from things like an isotope label to peptides to steroids to some non-ribosomal peptide derivative, or a fluorophore. So this has been used as a tool.

And you might ask, why is this possible? And if we just take a look at the structure of Sfp from B. subtilits with coASH and magnesium bound. What we see is that this end of the coA is extended out into the solvent. And at least in this structure here, it's not interacting with regions of the protein. So you can imagine that it's possible to attach some group, even a bulky group, here and be able to transfer it there.

So where we're going to focus the rest of the lecture is on an assembly line responsible for the biosynthesis of a natural product called enterobactin, and this is a siderophore. And so in thinking about this, what I would just like to first note is that when we talk about these assembly lines, we can group them into two types, which are non-iterative and iterative assembly.

And so what does this mean? So we've seen examples of non-iterative assembly last time on Monday with the ACV tripeptide and the vancomycin synthetase. So in these non-interative assembly lines, effectively, each step has its own module. So each carrier protein, T domain, each condensation or catalytic domain, is used only once as the chain grows. And we see the chain passed along from module to module here.

So also, the PKS we looked at for synthesis of DEB is one of these non-iterative assembly lines. So in contrast, in the example we're going to look at today with the enterobactin synthetase is an iterative assembly line, and this is similar to what we saw in fatty acid synthase. So in these iterative assembly lines, effectively, only one module is employed over

and over again.

So you can have the same carrier protein and same catalytic domain used for multiple cycles of chain elongation. And that's what we saw in fatty acid synthase, where there are multiple cycles in addition of a C2 unit via the same domains. And so what we're going to see today is this type of iterative assembly is responsible for the synthesis of this molecule here.

So first, just an overview of building blocks. And then we'll talk about why organisms want to make this molecule, and then focus on the biosynthetic logic and experiments. So this molecule, enterobactin, is produced from two monomers. So we have 2, 3 dihydroxybenzoic acid, or DHB, and we have serine here. And there is a two-module assembly line responsible for the synthesis of this natural product. And that assembly line is shown here.

So we see that there's three proteins, EntE, EntB, and EntF. We have an initiation module, elongation module, and this TE domain for termination. So overall, three separate proteins, two modules, and seven domains. So this NRPS is quite small. And this is an example of a non-ribosomal peptide that's produced by E. coli. So E. coli makes this molecule, as well as some other gram-negative bacteria.

So this is iterative. We have three of each of these monomers, yet only two T domains here, so imagine one responsible for each. So before we get more into this biosynthetic logic, let's just take a moment to think about why this molecule is produced. So this is a case where we actually have very good understanding about why an organism is producing a natural product. And this actually gives a segue into JoAnne's section on metal homeostasis, which will come up after cholesterol after spring break.

So many bacteria use non-ribosomal peptide synthesis machinery in order to make chelators in order to acquire iron. And that's because iron is an essential nutrient and it's actually quite scarce. So if you imagine an organism in the soil, maybe it needs to obtain iron from a rock. Somehow it needs to get iron from our pool, and concentrations are very tightly regulated, and most iron is tightly bound.

And we can also think about this from a standpoint of solubility, so simple KST type things. We all know that iron 3, which is the predominant oxidation state in aerobic conditions, is very insoluble. So our cars rust up here in the Northeast because they sit outside on the road in the winter, and that's no good. So we can think about 10 to the minus 18 molar.

And then if we think about free ion in human serum, for instance, the concentration is even lower because there's inherent toxicity associated with free iron. And you'll hear about that from JoAnne in more detail later. So these organisms have a predicament because for metabolism, they need iron on the order of micromolar concentrations.

So how does some organism obtain micromolar iron when in environments where, say, that's 10 to the minus 24 molar? And there's a number of strategies that come up, but one of the strategies is the biosynthesis of non-ribosomal peptides that act as metal scavengers and metal chelators. And so I just show you two examples here. And we have enterobactin, which we're going to focus on today. And this is really just a wonderful molecule.

Yersiniabactin-- and I put this up here, in part, because there were some questions about those cyclization domains in the bleomycin gene cluster, that we looked at that assembly line on Monday. And this is another example where cyclization of cysteine residues occurs in order to give the final natural product via those modified condensation domains here. So if we think about enterobactin for a moment longer, what happens, effectively, this molecule can bind iron 3 with higher affinity. And the iron bound form is shown here. So these aryl acids, these catechol groups, provide six oxygen donors to the iron center to get a structure like this.

So in terms of the organism in production, what happens when these organisms are confronted with iron limitations? So essentially, they're starved for essential nutrients. They'll turn on biosynthesis. So they'll express the enterobactin synthetase, which will allow for production of enterobactin. So this is happening in the cytoplasm. So we have those three proteins that comprise the assembly line that use the HDML serine to produce the natural product.

And then in addition to that biosynthetic machinery, the organism needs to also express and use a whole bunch of transport machinery. So what happens is that this natural product is exported into the extracellular space. So this is a gram-negative organism, so it has an inner membrane and an outer membrane. And it's in the extracellular space that enterobactin will scavenge iron 3.

So there's formation of the coordination complex, shown in cartoon here. And then there's a dedicated receptor on the outer membrane that will recognize that iron bound form and bring that into the cell. And then through transport and through breakdown of the natural product, this iron can be released and then used. So iron is a co-factor of many types of proteins and

enzymes here.

So a whole lot is going on. We're going to focus on the biosynthetic part. And so in thinking about this, from the standpoint of a non-ribosomal peptide synthetase, what's something interesting? So in the examples we saw last time, we had the ACD tripeptide, the vancomycin synthetase. These assembly lines are only forming peptide bonds, so we saw formation of amide bond.

If we take a look at enterobactin and we think about the monomers coming from, what do we see? So this has some C3 symmetry. And we can see that it's comprised of three of these DHB serine monomers, so 1, 2, 3. And effectively, there's formation of amide bonds between DHC and serine. So it's shown and throughout here. But there's also ester linkages formed. So this ring here is often called a trilactone, or a macrolactone, and somehow, these three esters need to be formed.

So how is the enterobactin synthetase doing this? So if we look at an overview of different enterobactin synthetase, the gene cluster, what do we learn? So the first point to make is that there are actually six proteins required. So you've seen three so far, in terms of the assembly line. So we have an A, B, C, D, E, and F. And A, B, and C are required for the biosynthesis of this aryl acid building block here, this DHB.

And then this is a case, rather unusual, where the PPTase was identified, and we're going to talk about that more as we go through the experiment. So I just told you about using Sfp if you don't know what to do. This was the case where the researchers were able to identify the dedicated PPTase for the assembly line. So that's EntE. And then we have B, E, and F that provide an iterative assembly line that yields the natural product, as shown here. OK, so also just note that B is coming up twice. We're seeing it here and we're seeing it here. So that should bring up a question, what's going on with this enzyme? And then we'll address that as we move forward.

So in terms of thinking about this synthetase, we'll do an overview and then look at the experiments. So we have an A domain and B. We have a protein here that has a T domain and an IDL domain that we'll get back to. This is EntE, and then here we have EntF. And then we have our PPTase. So effectively, here, we can have our initiation. Here, we have elongation. And here, termination.

So what is the overview, in terms of what happens for A domain activity? Loading of the T

domains and peptide bond formation. So for the overview, we'll first consider getting a monomer on to EntB. So EntB has a T domain. And that has a serine. The serine needs to be modified under the PPTase EntD. Holo EntD. We put the Ppant arm. And then what we'll see is that EntE is the A domain that's responsible for activating DHB and transferring that monomer to EntB.

So then in terms of EntF and getting the two domains of EntF loaded, it's going to be loaded with L-serine. And so here, you have EntF, again, focusing on the T domain. Again, we have that action of EntD to give us the holo form with the Ppant arm. And then we see that, in this case, the A domain is within the same protein. So the A domain of EntF is going to activate L-serine and transfer that to the T domain.

So we have EntF, A domain to get us here. So then what about peptide bond formation? So we see the C domain, condensation domain as an EntF. And so what we can imagine is that we have our EntB loaded with the aryl acid monomer plus EntF loaded with L-serine.

And what's going to happen? The C domain of EntF is going to catalyze formation of the amide bond here to give us EntB plus EntF, effectively, with DHA serine attached.

So this gives us some insight, just this overview, in terms of how the amide bond is formed and pretty much follows what we saw for the ACV tripeptide and vancomycin biosynthesis for the heptapeptide that forms its backbone. So a question we have at this stage is, well, we see in that structure, in addition to these amides, there's also esters. How are those formed? And then what assays are needed?

And so first, we're going to think about formation of the ester linkages, and then we'll launch into the experiment. So let's take a look at this assembly line. So we have EntE, the A domain, EntB, this didomain. That has the T domain. And here's EntF. And we see in this cartoon, the T domains are already modified with the P pant arm. And here is the serine residue of the TE domain that, ultimately, accepts the chain.

So what happens? If we take a look, so we saw this on the board, EntE becomes loaded with dihydroxybenzoic acid. EntF becomes loaded with serine. The condensation domain catalyzes this formation of an amide bond between two monomers. And then what happens? We see transfer of this DHB serine unit to the TE domain here. And then we can imagine these two domains being loaded with monomers again. And what happens?

What do we think about this? Effectively, formation of one amide bond transferred to the TE domain. Formation of another amide bond. And look. The second moiety here is transferred to the TE domain, to the initial monomer, via this ester linkage. This is really unusual behavior for a TE domain.

And what happens again? We see this happen again, so we get this linear trimer of enterobactin, effectively. And then what happens? Chain release to form the macrolactone here. We have this group that can come around here.

So what is the hypothesis? The hypothesis that was put forth by the researchers is that in this assembly line, effectively, this thioesterase is serving as a waiting room. And it's allowing these DHB serine monomers to wait around and remain attached, such that these esters can be formed. And somehow, it senses this appropriate size, this linear trimer, and then catalyzes chain release, as shown here.

AUDIENCE: Does it mess up?

ELIZABETH NOLAN: Does it mess up?

AUDIENCE: Yeah, does it always give you a 3 under [INAUDIBLE] circle?

ELIZABETH NOLAN: Yes, to the best of my knowledge. What's very interesting is that-- so this is worked out as Chris Walsh's group. Recently, Alison Butler's lab at Santa Barbara has discovered an enterobactin analog that has an additional unit in it here. So it looks like there's other thioesterases around that serve as waiting rooms and can accommodate different ring sizes. But this one will just give this size. And that's a very interesting question, just in terms of, how is this thioesterase doing that? We need more structural understanding to get at that.

In addition, these are just some overviews that I've put in the notes, other depictions of this process and the waiting room hypothesis from the literature. So we're going to look at the experiments that were done to study this. And I really, 1, like enterobactin, so I got excited about this molecule as an undergraduate, actually. But beyond that, why I like to present on this system, in terms of experiments, is that many firsts came from it, and it really serves as a paradigm for many, many other studies.

So if we just consider the various firsts that came from the studies of the enterobactin synthetase, 1, it was the first siderophore synthetase to be studied, and there's hundreds of

siderophores out there and many have been investigated since this one. It's the first example of a siderophore synthetase to be characterized for the Ppant arms. This was the first identification of a dedicated PPTase for one of these assembly lines. And the first identification of the thioesterase domain that has this behavior of forming this cyclo-oligomer. And the first identification of an aryl carrier protein, so this T domain that carries DHB here.

And in terms of the experiments we'll go through, these experiments that were devised in this system have been generalized across many, many assembly lines and the methods are still routinely used today. But a major difference I want to point out is that today, we have so many microbial genomes sequenced that a lot of work is driven by bioinformatics here, in terms of identifying that NRPS.

So often, the gene cluster may be identified well before the natural product is ever isolated. And this is a case where the natural product isolation occurred first, so that was done well, well before here. And this is a case where we know how to get the organism to produce this natural product. You starve the organism of iron and it will start to make it, in many instances, for other natural products produced by these assembly lines.

We don't know how to get the organism to actually make the molecule in a laboratory setting there. So there's some interesting work being done about that. Some actually recent work out of Northeastern, actually trying to grow organisms in soil-like environments and seeing what they can be provoked to produce. If you're curious, I'm happy to give you references.

OK, so where are we going to start, in terms of characterization of this synthetase here? We're, more or less, going to follow the logic outlined up here for this. So here's the cartoon of the players. And the first order of business is that it's necessary to characterize the adenylation domains. So we need to ask, what the monomers are selected and activated? And we have two adenylation domains to consider, so EntE and the A domain of EntF.

So what was done? For EntE, where we'll start, this protein was purified from E. coli and characterized here. And so how was it characterized? It was characterized by ATP-PPi exchange, like what we saw for the amino acyltransferase synthetase characterization. And so what was observed is that when EntE was combined with dihydroxybenzoic acid, ATP and radiolabeled PPi, there was incorporation of the P32 label into ATP. So that indicates formation of this adenylate intermediate. And resulted in the conclusion that EntE is the A domain that activates this aryl acid monomer, so this chemistry, which should be very familiar at this stage

based on our discussions in the translation unit.

So what about EntF and its A domain? So again, we're working with E. coli proteins. EntF was purified from E. coli. And again, this ATP-PPi assay was performed. And so in this case, what was observed is that when EntF was incubated with L-serine, ATP, and radiolabeled PPi, there was incorporation of the radiolabel into ATP, which indicates that EntF, its A domain is responsible for that activation of L-serine.

And so you can imagine in each set of experiments, the researchers also tried the other monomer, and in the case of EntF, would have seen no ATP-PPi exchange with DHB. And likewise, for EntE, if they tried with L-serine, there would be no exchange. You'd want to see that, in terms of making a robust conclusion here for that.

So that's good. Now, the next question is we need to get these monomers to these T domains here. And so that's the next step, is to study the T domain. And something you all need to appreciate about the time of this work, there wasn't a whole lot known about PPTases. There wasn't Sfp that you could borrow from your lab mate, or maybe you've expressed 100 milligrams for yourself and you could get that Ppant arm on here. And so JoAnne may want to elaborate, but there were a lot of effort to try to figure out, what is going on here?

JOANNE STUBBE: And graduate students had no thesis. Because they couldn't get any activity of any of the enterobactin genes.

ELIZABETH NOLAN: Yeah.

JOANNE STUBBE: Until it was discovered what was going on.

ELIZABETH NOLAN: Right. So this was a major, major effort, undertaking, and discovery here. And so they couldn't find activity, and that's because these two domains needed to be modified and they weren't modified. But some little detective work here. So in the analysis of purified EntF, what analysis of this purified protein had revealed, in some instances, with substoichiometric phosphopantetheine.

And so is that a contamination or is that meaningful? In this case, it was a meaningful observation that, when chased, proved to be very hopeful. It suggested that maybe there's a T domain here that's modified. That's something we can infer from this.

So if this Ppant arm is attached to EntF, how does it get there? And if we rewind and think about what was going on at the time, it was only shortly before that the PPTase for the acyl carrier protein and fatty acid synthetase was discovered. So that might beg the question, is it possible that this enzyme also modifies EntF, if you don't know much about its substrate scope? And so that hypothesis was tested and it didn't pan out. So if EntF was incubated with ACPS from fatty acid biosynthesis and coASH, there was no product formation. There was no transfer of the Ppant arm to here. Yeah?

AUDIENCE: Was it obvious the fatty acid synthesis-- was there [INAUDIBLE] synthesis at the time, or did it have a name?

ELIZABETH NOLAN: I don't think it had a name, but I defer to JoAnne, who was on the thesis committee, because this is really the first one.

AUDIENCE: Were the analogs of mercury obvious at the time?

ELIZABETH NOLAN: No, it's really discovery work at this stage. The question is, is there a possible lead from somewhere? And if you try it, what will happen? And really, there is no clue as to what is this modification and that design involved. But if you see an enzyme with activity in one system, maybe it will be active with another. Maybe not. And in this case, it didn't work, but it was something certainly worthwhile to try.

So then what was done? So there is a search for another PPTase, and this was done using BLAST. And what BLAST, this bioinformatics, revealed was the identification of that enzyme EntD. So here, we have this EntD, the PPTase here. And so EntD was overexpressed and purified. And so in this case, a histag was used, affinity column purification.

And the question is, what happens if we incubate EntD with EntF and coASH? And so in these experiments, radiolabeled coASH was used, and radio labels are commonly used to look for transfer of either Ppant arms or monomers, as we'll see as we go forward, to these domains. And so the question is, will we see transfer of the radiolabel to EntF in the presence of EntD and coASH?

And so here are the results from the experiment. So we have formation of holo EntF, as monitored by the radiolabel, versus time. And so what's done, the reaction is run for a given time point. The reaction is quenched with acid to precipitate the proteins. And then you can imagine measuring radioactivity in the pellet. coASH will remain in the soluble fraction and then

protein in the pellet here. You can imagine control assays with EntD included there.

And so what do we see? So as I said before, that we tried the acyl carrier, ACPS from fatty acid synthesis. There's no reaction. But look. When EntD is present, we see transfer of this Ppant arm to the protein here. So this was a really exciting result at the time. We have a new enzyme, a new activity, this post-translational modification there. And this opens the door to further studies. If you can get the Ppat arm on, then we can look at loading the monomers here.

So what's the next step? We have EntF loaded. We're also going to want to try to load EntD-- EntB, excuse me-- here. But of course, you need to know some more about EntB. And so let's think about that. I'll also note, just noted here, the next step is to look at loading of L-serine onto this moiety here, as drawn. And you can think about how to do that experimentally.

So what about EntB? This was another mystery, in terms of experimental work and exploration. And so initially, EntB was purified and characterized for its activity that led towards the biosynthesis of the BHP monomer. So this ICL domain is involved in the series of reactions that give DHB.

On a historical note, it was thought there was another protein. And this protein was named EntG that was thought to be required for enterobactin biosynthesis. And EntG would be the T domain that is for the aryl acid. So effectively, it would be this T domain, or aryl carrier protein for dihydroxybenzoic acid. But the problem was they couldn't find a gene for EntG.

And so as it turned out, what more detective work showed is that this EntG is actually just the N-terminus of EntB here. So they realize that EntB has another role, another function, and that in addition to having this function and the synthesis of dihydroxybenzoic acid, because of this domain at the N-terminus, it's also the carrier protein for this monomer here.

So how is this sorted out here? What we can do is just take a look at a sequence alignment. And so this is from one of the papers about all of these explorations. And effectively, what we're taking a look at are known [INAUDIBLE] phosphopantetheinylation sites, the proteins. So something is known about fatty acid synthesis and some other carrier proteins here from different organs.

And so effectively, if we just look at this region of the alignment, we see this serine residue with an [INAUDIBLE] And this happens to be serine 245 of EntB. So this led to the hypothesis that

maybe this serine 245 towards the C-terminus terminus of EntB is the site where the Ppant arm is attached here. And so this means that some experiments are needed to show that EntB has this carrier protein, or T domain, and that it can be modified with the Ppant arm. And it was predicted EntD would do this. And also, that once modified with the Ppant arm, the aryl acid can be transferred to EntD.

So if we just think about EntB for a minute, So have the N-terminal domain. Here's the C-terminal domain. Here's the ICL domain. Here's the T domain for an aryl carrier protein. So amino acid 1, 285. This is 188. It's not quite drawn to scale. So we serine 245 around here, which is the serine of interest for post-translational modification with the Ppant arm.

And so what was done is that pathways were performed, where EntB was incubated with EntE and radiolabeled coASH, like what we saw for the studies of EntF. But they made a few additional constructs. So they considered full length EntB, so as shown here. They considered an EntD variant where with C-terminal 25 amino acids were deleted. So you can imagine, they just put a stop codon in and leave the last 25 amino acids. So the serine is still there, but a bunch of the C-terminal residues aren't there. And then they also considered a variant of EntB where they deleted this entire N-terminal domain.

And so the question is, what are the requirements? Well 1, does this reaction work? Does EntD modify EntB with the Ppant arm? And then if yes, what are the requirements? So is the N-terminal domain needed? Are these C-terminal residues needed? And so these are the gels that come from this experiment. And so what we're looking at on top are total proteins, so Coomassie staining. And on the bottom, we're looking at radioactivity. And the idea here is that we want to track the radiolabels.

So in lane 1, we have assays with full length EntB. In lane 2 with the C-terminal truncation. And in lane 3, deletion of the N-terminal domain. OK, so the question is, what do we see from these data here? And so these give us a sense as to where the individual proteins run on the gel. And here, we're looking at the radioactivity.

So what do we see? In lane 1, you see a huge blob of radioactivity. This isn't the most beautiful gel, actually. Nevertheless, there's much to learn. So what do we see? We see radioactivity associated with EntB. That's really good news. We see transfer of this radiolabeled Ppant arm. What about lane 3? So what do we see there?

AUDIENCE: Also a lot of radioactivity.

ELIZABETH NOLAN: Right. We have a lot of radioactivity. We're looking at the construct that only has this C-terminal domain. So what does that tell us?

AUDIENCE: It's shorter. That's why it moved down the gel further.

ELIZABETH NOLAN: Right. So that's why it has a different migration on the gel. But in terms of seeing the radioactivity, what did we learn? Is this region of the protein essential or dispensable? We don't need this N-terminal domain in order for EntD to modify EntB. So we're seeing that. What about in the middle?

AUDIENCE: The deleted region is important [INAUDIBLE]

ELIZABETH NOLAN: Right. We see very little radioactivity here. Basically, almost nothing, especially compared to these spots. So deletion of those C-terminal amino acids is detrimental, and so that region is important. So maybe there's protein-protein interaction going on, or something with information that's important. So from here, we learn that EntD transfers the Ppant arm to EntB. The ICL domain is not essential for this, but the C-terminus of this region is here.

So now what? We've got in here via the action of EntD. Can we get attachment of the monomer? And so our hypothesis is that EntE, which we saw EntE activate DHB to form with the adenylate, it will also transfer this moiety to EntB. So in this case, what was done, again, we're looking at use of a radiolabel. In this case, the radiolabel is on the DHB lane.

And this is an important point. In order to see this, we cannot have radiolabeled Ppant arm, in this case, because that would give you a big background. So they're going to prepare EntB with the Ppant arm unlabeled. We know that will work based on the prior study. And now, we repeat that with unlabeled coASH. And then ask, if we incubate total EntB with EntE, ATP, and radiolabeled DHB, do we see transfer of the radiolabel to this protein here?

JOANNE STUBBE: Let me ask a question. This will be a question during class. Can you do this experiment with tritiated CoA and C14-labeled serine, based on what you know about radioactivity? We actually discussed a similar situation.

AUDIENCE: Would it last longer [INAUDIBLE]

JOANNE STUBBE: Did you go back and look at the lifetimes? Is it infinite compared to any experiments? So it's not lifetimes. Do you have any ideas?

AUDIENCE: I mean so tritium, the energy, the particle released, is much lower than the energy of C14.

JOANNE STUBBE: Right. So the difference is in the energies. We talked about this. You can tune the scintillation counter. So you measure tritium to C14. So people that do enzymology for a living often use tritium in C14 at the same time. And you can quantitate, if you do your experiments right. It's a very powerful tool together, actually.

ELIZABETH NOLAN: So another option, the non-simplistic approach. So basically here, if we're looking at the four lanes, again, we're looking at total protein. We're looking at radioactivity, and can consider the overall reaction, and then a variety of controls. OK, and I want to move forward to get through the rest of the slides and we just have a few more minutes, but you should work through this gel and convince yourself that there is transfer of this radiolabel in the presence of EntD And this was done with unlabeled EntD.

OK, so what about peptide bond formation? We have the T domains loaded with the monomer. Can we see activity from the C domain, in terms of the formation of an amide bond. And so this experiment requires a lot of components. So what is the experiment? To look at whether or not EntF catalyzes condensation. Basically, we can incubate EntE, holo EntE, holo EntF, ATP, and these monomers.

And what we want to do, in this case, is look at transfer of radiolabeled DHB to serine-loaded EntF. And I guess I got a little ahead of myself on the prior slide. So this is a case where if you add C14 labels in both of your monomers, you'd have a big problem. So key here is to use unlabeled serine and radiolabeled DHB so you're not getting a big background.

And an important point to make here in these experiments is that we're looking for detection of a covalent intermediate. So effectively, having this guy here attached to EntF. And so the radiolabel is here. So that's what we're looking for, not the final product, and that's indicated by having the gels.

So what do we see? We have the total protein and then the autoradiograph. And so we have holoEntF, EntE, holoEntB. And the question is, where do we see radiolabels transfer? And if we look at lane 3, where we have the proteins, ATP, serine, and radiolabeled DHB, what we observe is that there is some radioactivity here, which is indicative of a covalent intermediate. And again, you should work through these gels and work through the different conditions and make sure it makes sense to you what's seen in each one.

So finally, at that stage, the activities for all of these different domains have been found. And so the question is, in the test tube, can we actually get enterobactin biosynthesized, which is going to rely on this TE domain. So the idea is if we incubate everything together, similar to what was done before, can we detect the actual small molecule, rather than this intermediate attached to EntF here?

And so the way this was done was by monitoring the reaction by HPLC using reverse stage chromatography. And so here, we have all of the reaction components. Here, we see standard. So in enterobactin, this is the linear trimer, the linear dimer, a monomer. Here's the DHB substrate. And the question is, over time, do we see enterobactin formed? So you can imagine quenching the reaction, taking the soluble component, which should have this small molecule, and looking at each POC.

And where you should just focus at the moment is here. So the enterobactin peak, what we see is that over time, there's growth in this peak. You can imagine doing something like LC-MS analysis to confirm it is the species you expect here.

So this is full in vitro reconstitution of a non-ribosomal peptide synthetase in the test tube, and it really paved the way for many, many additional experiments related to these types of biosynthetic machines. And so with that, we're going to close this module, and I hope you all have a great spring break. And I leave you in the good hands of JoAnne starting the 28th here for lecture.