Steps, some of them independent, for creating a alcohol-producing JH1140 mutant

A. Create a culture of JH1140 from the lyophilized sample.
There are instructions about recommended culture media in the "Culturing from Dental Samples" document, though I'm not confident those are the best methods / media.

B. Demonstrate an assay for lactic acid production.
(*depends on* A)

Assay the culture for lactic acid production. There are probably standard assays for this, and some might be described in Hillman's papers. My guess is you might be able to use some kind of pH metric, but I'm not sure. Regardless, you want to establish some correlation between amount of JH1140 x amount of sugar = amount of lactic acid. You could compare a control plate (no JH1140) and cultures are varying ODs (or varying sugar concentrations, keeping OD constant) and then assay.

C. Demonstrate an assay for alcohol production.
(*weak dependency on* B)

Not sure the best way to test this. Add trace concentrations of ethanol to media? At some point, we need to make sure that this assay doesn't interfere with the lactic acid assay.

i. Investigate CRISPR Prime for this application.
I think it might be, and should create less risk of off-targeting, but I don't know how much you can just buy it or if it's been used in gram-positive bacteria. This is basically a literature review task, plus checking AddGene and wherever to see if you can get it.

ii. Demonstrate transformation of S. *mutans*.
(*weak dependency on* A)

Depending on how well JH1140 is behaving, you could use it or UA159 (the reference strain). Transform a fluorescing plasmid into an S. *mutans* strain. I'm sure there are protocols for this in the literature. In addition, demonstrate ability to genetically verify that transformation has occurred. This will involve a miniprep and then either a restriction digest + fragment analysis (e.g. gel electrophoresis) or you can tag the DNA and send it off to IDT for NGS (it doesn't *have* to be IDT, but they're big). Honestly, probably do both of those things and check that they produce the same result. You might be able to copy some protocols from iGEM teams here?

iii. Demonstrate functionality of selected CRISPR mutagenesis system in S. *mutans*.
(*Depends on i*, *ii*)

First, you need to design a construct. This is going to involve the plasmid with a positive selection marker + a fluorescent marker that you presumably used in step *ii*. Add Cas9 (or whatever version of it works best in S. *mutans*, which is a question for the literature i.e. you figure this out in step *ii*) to the fluorescent plasmid (I'm going to call this construct "coCas".) You should also design a guide RNA that targets a fluorescent marker.

Then test and compare:
1. Base strain (UA159 or JH1140)
2. Strain w/ coCas and no guideRNA
3. Strain w/ coCas and a guide RNA that targets a second fluorescent marker
4. Strain w/ coCas and a guide RNA that targets some random sequence

1. Produce a construct with the modified pheS negative selection marker.
(*weak dependency on ii*)

This is the marker described in "*Recombineering in Streptococcus mutans Using Direct Repeat-Mediated Cloning-Independent Markerless Mutagenesis (DR-CIMM)*". It's *pheS* from *E. Coli* plus T260S + A314G + some silent mutations downsteam from A314 that prevent recombining with the wild type. (More details on this in the "WB_Protocol" document, there's a section called "pheS Counterselection".)

After producing the modified pheS you should connect it to something that will make assembly easy. Maybe use the gBlocks standard from IDT? This is probably a short enough sequence that you could just order a moderately expensive gBlock with the full sequence. Those are designed for Gibson assembly. If you want to do another kind of assembly (golden gate? restriction digests?) you will need a different fragment design. This fragment design will depend on what is easily integrated to whatever kind of plasmid you showed you could transform in step *ii*.

2. Demonstrate that ePheS can work as a negative selection marker in S mutans.
(*depends on 1,*)

That is, put it on a plasmid with an antibiotic resistance gene and a fluorescent marker. First, plate on media with antibiotic. Then, select surviving colonies and plate on media with 4CP. Check that some colonies survive, and that these are not fluorescent (compare with control plate, obvi). Using the transformation verification techniques from step *ii* (digests or whatever) verify that the surviving colonies have lost the plasmid, Make sure to contrast several biological biological replicates from control plates.

iv. Create an insert for alcohol dehydrogenase.
(*depends on iii*)

This should be the ADH enzyme we want plus homology arms for the region around LDH. I think just straight up linearized DNA will work as a final product, but it may end up being cheaper / easier to do PCR OE or some kind of assembly than to just order a fully synthesised fragment. Even if we order that fragment, we should probably PCR up a bunch of copies and do some kind of sequence confirmation.

The sequence needed to duplicate Hillman's work (without complete homology arm sequences) is described in "WB_Protocol". The kind of homology arm we want will depend a whole lot on what CRISPR system we're using.

v. Design guide RNAs to insert the insert.
(*depends in iv*)

This is vague because, again, it depends a lot on the chosen CRISPR system. But, yeah sgRNAs or whatever the appropriate analogue is called to match the place where we want to insert the insert designed in the previous step.

vi. Design complete CRISPR construct.
(*depends on v, 2*)

Design CRISPR plasmid containing Cas system, guide RNAs and positive + negative selection markers. Construct this plasmid and sequence confirm it.

At this point, we have a linear series of steps that depend on everything that came before. They're a bit vague, because you're going to learn things during those steps:

Transform the CRISPR construct into JH1140.

Demonstrate growth, successful transformation, lactic acid production, and lack of alcohol production. *Maybe* we should also have some kind of assay for mutacin 1140 activity? Let's call this strain WB1140.

Add adh sequence.
(This might be compressed into the previous step if using CRISPR Prime).

Use an unrelated linear fragment as a control. Move on once we have a culture that produces alcohol and not lactic acid.

Use negative selection to remove CRISPR plasmid.
Confirm that one of the resulting strains produces alcohol and not lactic acid. Sequence confirm for presence of *adh* gene, lack of *ldh* gene, and lack of various markers from the CRISPR plasmid (i.e. make sure it didn't *just* mutate the *mPheS* gene).

Grow an oral culture of S.*mutans*.
Assay for lactic acid production and lack of alcohol production. Sequence confirm sequence for ldh gene. (This is to confirm that we'll be able to measure successful colonization.)

Attempt colonization of small number of volunteers. Wait a few months.

Culture oral samples from volunteers.
Assay to confirm alcohol production and lack of lactic acid production.

Distribute strain to larger number of volunteers.